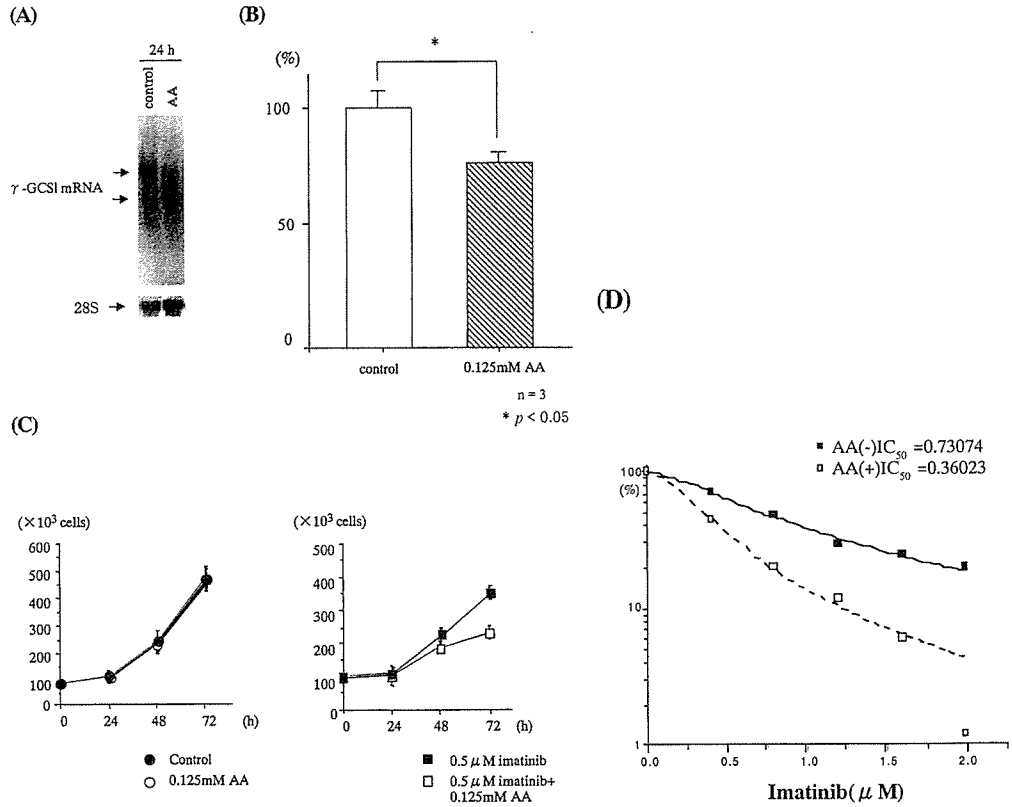

Figure 5. Effect of AA on GSH synthesis in KCL22/SR cells. **(A,B)**: KCL22/SR cells were cultured in the presence of 0.125 mM AA for 24 hours. Changes in the levels of γ-GCSl mRNA **(A)** and GSH concentration **(B)** were examined as described in Materials and Methods. **(C)**: KCL22/SR cells were incubated with or without 0.125 mM AA in the absence or presence of 0.5 μM imatinib for 72 hours. Viable cells were counted by trypan blue exclusion at various time points, as indicated in the figure. **(D)**: KCL22/SR cells were incubated in the presence of various concentrations of imatinib with or without ascorbic acid. The IC_{50} values of imatinib are shown in the figure.

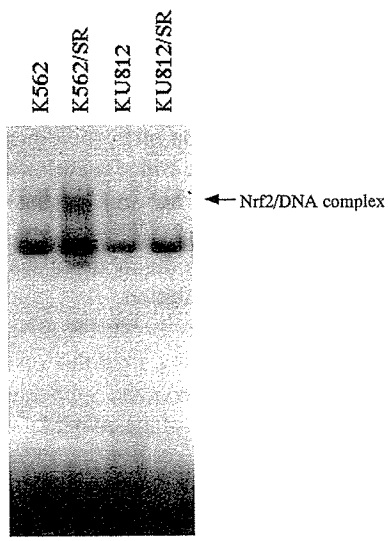

Figure 6. Formation of Nrf2/DNA complex at ARE in other imatinib-resistant cell lines. Nuclear extracts were prepared from K562, K562/SR, KU812, and KU812/SR cells. Gel mobility shift assay was performed using the end-labeled oligomers corresponding to ARE.

imatinib treatment (data not shown). We did not examine changes in Nrf2/DNA complex formation induced by AA in that case; however, it is possible that AA-induced inhibition of Nrf2 activity strengthened the effect of imatinib. It has been reported that reactive oxygen species could inhibit the activity of protein tyrosine phosphatases, resulting in the induction of protein tyrosine phosphorylation [32]. Thus, AA may also have other biological effects through phosphatase activation. Taken together, these results suggest that AA is an attractive molecular target reagent for overcoming resistance to imatinib in some imatinib-resistant CML cells.

Acknowledgments

This work was supported in part by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology, Japan. We wish to thank Ms. E. Yamakawa for her help in preparation of the manuscript.

References

1. Druker BJ, Talpaz M, Resta DJ, et al. Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. N Engl J Med. 2001;344:1031–1037.

2. Druker BJ, Sawyers CL, Kantarjian H, et al. Activity of a specific inhibitor of the BCR/ABL tyrosine kinase in the blast crisis of chronic myeloid leukemia and acute lymphoblastic leukemia with the Philadelphia chromosome. N Engl J Med. 2001;344:1038–1042.
3. Sawyers CL, Hochhaus A, Feldman E, et al. Imatinib induces hematologic and cytogenetic responses in patients with chronic myelogenous leukemia in myeloid blast crisis: results of a phase II study. Blood. 2002;99:3530–3539.
4. le Coutre P, Tassi E, Varella-Garcia M, et al. Induction of resistance to the Abelson inhibitor STI571 in human leukemic cells through gene amplification. Blood. 2000;95:1758–1766.
5. Weisberg E, Griffin JD. Mechanism of resistance to the ABL tyrosine kinase inhibitor STI571 in BCR/ABL-transformed hematopoietic cell lines. Blood. 2000;95:3498–3505.
6. Mahon FX, Deininger MW, Schultheis B, et al. Selection and characterization of BCR-ABL$^+$ cell lines with differential sensitivity to the tyrosine kinase inhibitor STI571: diverse mechanisms of resistance. Blood. 2000;96:1070–1079.
7. Gorre ME, Mohammed M, Ellwood K, et al. Clinical resistance to STI571 cancer therapy caused by BCR-ABL gene mutation of amplification. Science. 2001;293:876–880.
8. Branford S, Rudzki Z, Walsh S, Grigg A, Arihur C, Taylor K. High frequency of point mutations clustered within the adenosine triphosphate–binding region of BCR/ABL in patients with chronic myeloid leukemia or Ph$^+$ acute lymphoblastic leukemia who develop imatinib (STI571) resistance. Blood. 2002;99:3472–3475.
9. Gambacorti-Passerini C, Barni R, le Coutre P, et al. Role of α1 acid glycoprotein in the in vivo resistance of human BCR-ABL$^+$ leukemic cells to the Abl inhibitor STI571. J Natl Cancer Inst. 2000;92:1641–1650.
10. Venugopal R, Jaiswal AK. Nrf1 and Nrf2 positively and c-Fos and Fra1 negatively regulate the human antioxidant response element–mediated expression of NAD(P)H:quinone oxidoreductase1 gene. Proc Natl Acad Sci U S A. 1996;93:14960–14965.
11. Venugopal R, Jaiswal AK. Nrf2 and Nrf1 in association with Jun proteins regulate antioxidant response element–mediated expression and coordinated induction of genes encoding detoxifying enzymes. Oncogene. 1998;17:3145–3156.
12. Itoh K, Wakabayashi N, Katoh Y, et al. Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. Genes Dev. 1999;13:76–86.
13. Itoh K, Chiba T, Takahashi S, et al. An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. Biochem Biophys Res Commun. 1997; 236:313–322.
14. Ishii T, Itoh K, Takahashi S, et al. Transcription factor Nrf2 coordinately regulates a group of oxidative stress-inducible genes in macrophages. J Biol Chem. 2000;275:16023–16029.
15. Richman PG, Meister A. Regulation of γ-glutamyl-cysteine synthetase by nonallosteric feedback inhibition by glutathione. J Biol Chem. 1975; 250:1422–1426.
16. Meister A. Glutathione metabolism. Methods Enzymol. 1995;251:3–7.
17. Filomeni G, Rotilio G, Ciriolo MR. Glutathione disulfide induces apoptosis in U937 cells by a redox-mediated p38 MAP kinase pathway. FASEB J. 2003;17:64–66.
18. Kong AN, Yu R, Lei W, Mandlekar S, Tan TH, Ucker DS. Differential activation of MAPK and ICE/Ced-3 protease in chemical-induced apoptosis: the role of oxidative stress in the regulation of mitogen-activated protein kinases (MAPKs) leading to gene expression and survival or activation of caspases leading to apoptosis. Restor Neurol Neurosci. 1998;12:63–70.
19. Haddad JJ. Redox regulation of pro-inflammatory cytokines and IκB-α/NF-κB nuclear translocation and activation. Biochem Biophys Res Commun. 2002;296:847–856.
20. Hayes JD, Pulford DJ. The glutathione S-transferase supergene family: regulation of GST and the contribution of the isoenzymes to cancer chemoprotection and drug resistance. Crit Rev Biochem Mol Biol. 1995;30:445–600.
21. Iida T, Mori E, Mori K, et al. Co-expression of γ-glutamylcysteine synthetase sub-units in response to cisplatin and doxorubicin in human cancer cells. Int J Cancer. 1999;82:405–411.
22. Takemura H, Urasaki Y, Yoshida A, Fukushima T, Ueda T. Simultaneous treatment with 1-β-D-arabinofuranosylcytosine and daunorubicin induces cross-resistance to both drugs due to a combination-specific mechanism in HL60 cells. Cancer Res. 2001;61:172–177.
23. Moinova HR, Mulcahy RT. An electrophile responsive element (EpRE)regulates β-naphthoflavone induction of the human γ-glutamylcysteine synthetase regulatory subunit gene. Constitutive expression is mediated by an adjacent AP-1 site. J Biol Chem. 1998;273:14683–14689.
24. Kubonishi I, Miyoshi I. Establishment of a Ph1 chromosome–positive cell line from chronic myelogenous leukemia in blast crisis. Int J Cell Cloning. 1983;1:105–117.
25. Ohmine K, Nagai T, Tarumoto T, et al. Analysis of gene expression profiles in an imatinib-resistant cell line, KCL22/SR. Stem Cells. 2003; 21:315–321.
26. Anderson ME, Levy EJ, Meister A. Preparation and use of glutathione monoesters. Methods Enzymol. 1994;234:492–499.
27. Nagai T, Tarumoto T, Miyoshi T, et al. Oxidative stress is involved in hydroxyurea-induced erythroid differentiation. Br J Haematol. 2003; 121:657–661.
28. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem. 1987;162:156–159.
29. Nagai T, Harigae H, Ishihara H, et al. Transcription factor GATA-2 is expressed in erythroid, early myeloid, and CD34$^+$ human leukemia–derived cell lines. Blood. 1994;84:1074–1084.
30. Lassar AB, Davis RL, Wright WE, et al. Functional activity of myogenic HLH proteins requires hetero-oligomerization with E12/E47-like proteins in vivo. Cell. 1991;66:305–315.
31. Nagai T, Harigae H, Furuyama K, et al. 5-aminolevulinate synthase expression and hemoglobin synthesis in a human myelogenous leukemia cell line. J Biochem (Tokyo). 1997;121:487–495.
32. Sattler M, Verma S, Shrikhande G, et al. The BCR/ABL tyrosine kinase induces production of reactive oxygen species in hematopoietic cells. J Biol Chem. 2000;275:24273–24278.

THE JOURNAL OF GENE MEDICINE
J Gene Med 2004; 6: 288–299.
Published online 28 January 2004 in Wiley InterScience (www.interscience.wiley.com). **DOI:** 10.1002/jgm.490

RESEARCH ARTICLE

In situ generation of pseudotyped retroviral progeny by adenovirus-mediated transduction of tumor cells enhances the killing effect of HSV-*tk* suicide gene therapy *in vitro* and *in vivo*

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Received: 3 March 2003
Revised: 24 July 2003
Accepted: 2 August 2003

Abstract

Background Hybrid adeno-retroviral vector systems utilize the high efficiency of adenovirus transduction to direct the *in situ* production of retroviral progeny. In this study, we show that a single-step transduction of glioma cells with trans-complementing hybrid adeno-retroviral vectors effectively turns these cells into retrovirus vector-producing cells, which in turn facilitates the transduction of adjacent cells.

Methods We have adapted the adeno-retroviral hybrid viral vector system to enhance the ganciclovir (GCV) killing of glioma cells following transfer of the herpes simplex virus thymidine kinase (HSV-*tk*) gene. To assess the effect of the *in situ* production of retroviral vectors on the transduction efficiency of glioma cells, 9L cells were transduced with adeno-retroviral hybrid vectors that separately express a retroviral genome (AVC2.GCEGFP or AVC2.GCTK) and retroviral packaging proteins (AxTetGP and AxTetVSVG). The generation of an integrated HSV-*tk* provirus by trans-complementation of the adeno-retroviral vectors was verified by analysis of the flanking retroviral LTR sequences. Tumors established on *nu/nu* mice were injected with the viruses followed by intraperitoneal injections of either PBS or GCV. We also estimated the copy numbers of the HSV-*tk* transgene present in the tumors of the treated mice. To determine the expression pattern of the HSV-*tk* transcripts within a tumor, *in situ* hybridization analysis was performed using an RNA probe specific for HSV- *tk*.

Results The co-transduction of rat 9L glioma cells with AVC2.GCEGFP together with vectors expressing packaging proteins of retroviruses increased the transduction efficiency. Transduction with AVC2.GCTK together with packaging vectors increased the *in vitro* sensitivity of cells to the pro-drug GCV by one log compared with control cells that were incapable of generating retrovirus. *In vivo*, the injection of established subcutaneous 9L tumors on athymic mice with a combination of AVC2.GCTK and packaging vectors followed by GCV treatment resulted in complete tumor regression in 50% of tumors at day 22, while no tumor regression was observed in control animals. Retroviral sequences diagnostic of 3′ LTR reduplication *in vivo* were detected in genomic DNA extracted from the transduced tumors, indicating pro-viral integration of the retroviral genome derived from the adeno-retroviral hybrid vector. Furthermore, the relative copy number of the HSV-*tk* gene in tumors treated with the adeno-retroviral vectors was up to ~250-fold higher than in control tumors. *In situ* hybridization suggested dispersion of the HSV-*tk*

product across a wider area of the tumor than in control tumors, which indicates the spread of the *in situ* generated retroviruses.

Conclusions Although the efficacy of this system has to be evaluated in orthotopic models, our observations suggest that this hybrid adeno-retroviral vector system could improve the suicide gene therapy of tumors. Copyright © 2004 John Wiley & Sons, Ltd.

Keywords suicide gene therapy; adeno-retroviral hybrid vector; *in situ* generation; glioma; HSV-*tk*

Introduction

When tumor cells that have been transformed with the herpes simplex virus thymidine kinase (HSV-*tk*) gene are treated with the pro-drug ganciclovir (GCV), they regress [1]. However, the clinical benefit of this cancer gene therapy system is limited due to the poor efficiency of gene transfer [2]. To improve the therapeutic potential of this system, it is necessary to enhance the efficiency of the delivery of a therapeutic gene *in vivo* as well as to increase the stability of the expression of the gene.

We and others have previously described hybrid vector systems that use adenoviral vectors to deliver retroviral vector and packaging proteins into cells [3–9]. These systems benefit from the efficient gene transfer characteristics of adenoviral vectors as well as from the stable and long-term gene expression that is typical of retroviral vectors. The initial co-transduction of such adeno-retroviral hybrid vectors results in the transient production of recombinant retrovirus particles that then subsequently transduce neighboring cells. Adenovirus vectors expressing trans-complementing genes for retroviral proteins and retroviral vector RNAs have been successfully used for the *in situ* transduction of tumor cells [3,7].

We describe here an improved adeno-retroviral vector system that efficiently produces pseudotyped retroviral vectors. To simplify the construction of the chimeric vector carrying the retroviral genome, the directional ligation technique with the DNA–protein complex was employed [10]. We show that our adeno-retroviral packaging system can be used to rescue an integrated retroviral provirus in tumor cells and to enhance the therapeutic gene expression in glioma cells. In addition, we demonstrate that an adeno-retroviral HSV-*tk* vector system coupled with GCV enhances GCV-mediated killing of glioma cells both *in vitro* and *in vivo*. This suggests that this strategy produces sufficient levels of vectors *in situ* for the killing of solid tumors.

Materials and methods

Plasmid construction

The hybrid adeno-retroviral vector plasmid pAVC2.GCE-GFP was constructed as follows. The 3′ long terminal repeat (LTR) (*Cla* I-*Nde* I fragment) of the Molony murine leukemia virus (MMLV)-based retroviral vector pGCsap was replaced with the 3′ LTR (*Cla* I-*Nde* I fragment) of the myeloproliferative sarcoma virus (MPSV) to form pGCsapM. To generate pGCsapMEGFP, the *Nco* I-*Not* I fragment from pEGFP-N1 (Clontech Laboratories, Palo Alto, CA, USA) containing cDNA encoding the enhanced green fluorescent protein (EGFP) was cloned into *Nco* I-*Not* I-digested pGCsapM. This ensured that the EGFP translational initiation site was precisely located at the *env* translational start site found in the wild-type MMLV retrovirus. The retroviral vector GCsapMEGFP cassette was then cloned as an *Asc* I–*Xba* I-fragment into *Asc* I–*Xba* I-digested pAVC2.LXSN [5], from which the LXSN retroviral vector genome had been removed, to generate pAVC2.GCEGFP (Figure 1A).

The hybrid adeno-retroviral vector plasmid pAVC2.GC-TK was derived from pGCsam [11]. The full length coding region of the HSV-*tk* cDNA contained in the *Spe* I-*Cla* I fragment from pAVS6TK [12] was subcloned into pBluescript SK (+) and then cloned as a *Not* I–*Cla* I fragment into *Not* I–*Cla* I-digested pGCsam, which generated pGCsamTK. To construct pAVC2.GCTK (Figure 1A), the retroviral vector GCsamTK cassette was cloned as an *Asc* I–*Xba* I fragment into *Asc* I–*Xba* I-digested AVC2.LXSN.

Generation of adenovirus vectors

The recombinant hybrid adeno-retroviruses AVC2.GCE-GFP and AVC2.GCTK were generated using the mutant adenovirus type 5, dl327 [13]. A DNA–protein complex of dl327 was prepared as described previously [10]. The dl327 DNA–protein complex was digested with *Cla* I and *Bst* Z17 and treated with bacterial alkaline phosphatase (LTI, Grand Island, NY, USA) at 37 °C for 30 min. The enzymes were removed by three cycles of dilution with TE (pH 8.0) followed by concentration by using a Centricon YM-100 column according to the manufacturer's instructions (Amicon, Beverly, MA, USA). The AVC2.GCEGFP and AVC2.GCTK cassettes were excised from their respective plasmids (pAVC2.GCEGF and pAVC2.GCTK) by *Rca* I and *Bst* Z17 digestion and purified by agarose gel electrophoresis (QIAquick gel extraction kit; Qiagen, Hilden, Germany). The purified

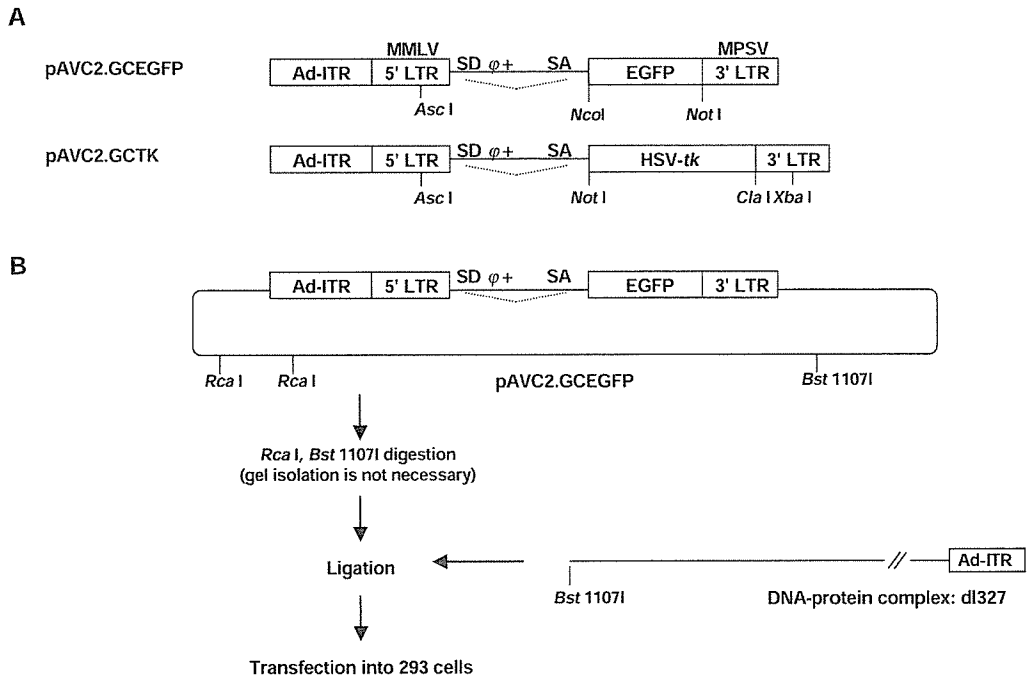

Figure 1. Schematic representation of the adeno-retroviral hybrid vectors used in this study. (A) The adeno-retroviral hybrid vector plasmids containing retroviral vector genomes expressing either GFP (AVC2-GCEGFP) or HSV-*tk* (AVC2-GCTK). SD, splice donor; SA, splice acceptor. (B) Cloning of recombinant adeno-retroviral hybrid vectors using directed ligation of a linearized vector plasmid into a DNA–protein complex in a single step without homologous recombination

fragments were mixed with 0.2 μg of *Cla* I- and *Bst* Z17-digested dl327 DNA–protein complex (Figure 1B) at a molar ratio of 3 : 1 and ligated with 5 Weiss U of T4 DNA ligase (New England Biolabs, Beverly, MA, USA) in a volume of 10 μl overnight at 9 °C. The ligated samples were desalted by using a Centricon YM-100 column and transfected into 293 cells by calcium phosphate co-precipitation, after which the cells were overlaid with agar as previously described [14]. The infectivity of the ligated DNA–protein complex was approximately 10 plaque-forming units/μg of DNA–protein complex for both vectors. Virus plaques were isolated and expanded in 293 cells. All viruses screened by PCR for the presence of the inserted retrovirus vector genome were positive. A control adenovirus vector AVC2.null with no expression gene cassette was constructed and propagated as described before [10]. The adenoviruses AxTetGP and AxTetVSVG expressing the MMLV gag-pol and the G protein of vesicular stomatitis virus (VSV-G) envelope protein, respectively, have been described previously [15]. The expression of the gag-pol and VSV-G proteins is under the control of the transcriptional regulator reverse Tet-controlled transactivator (rtTA) that is supplied by the adenoviral vector AV-rtTA [7]. These adenoviral vectors were propagated and purified as described previously [14].

Cell lines

The amphotropic retroviral vector producer cell line FLYA13 [16], the rat glioma cell line 9L [17], the human glioma cell line D54 [18], and the human embryonic kidney cell line 293 [19] were cultured in Dulbecco's modified Eagle's medium (DMEM high glucose; 4.5 g/l, Life Technologies, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS, Life Technologies), 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine at 37 °C, 10% CO_2. To express the transgene from AxTetGP and AxTetVSVG, the cell culture medium was supplemented with 1 μg/ml doxycycline (Sigma, St. Louis, MO, USA). The GFP reporter cell line D54GFPneo was established by transfecting the retroviral expression plasmid pGC-GFP-loxP-EN [20] into D54 cells by calcium phosphate co-precipitation [21] followed by selection of independent GFP-positive colonies with 0.5 mg/ml G418 (Life Technologies).

Viral transductions

The cells were transduced with adenoviruses for 3 h at a variety of multiplicities as described for each experiment. The cells were washed with growth medium supplemented with 1 μg/ml of human serum γ-globulin (Miles Research Products Division, Elkhart, IN, USA) as a source of neutralizing antibody against adenovirus (these washes significantly reduce the carryover of residual adenoviral vectors in newly synthesized retroviral supernatants [5]). Finally, growth medium supplemented with human serum γ-globulin (1 μg/ml) and doxycycline (1 μg/ml) was added to each flask.

Putative retroviral-containing supernatants were harvested at the indicated time and centrifuged at 700 *g*, 4 °C,

filtered (0.45 μm), aliquotted and stored at −80 °C. For retroviral transduction, target cells were plated in 6-well plates at 5×10^4 cells per well and incubated overnight at 37 °C in 5% CO_2. Duplicate retroviral transductions were conducted at 37 °C by plating 2 ml of undiluted viral supernatant supplemented with polybrene (8 μg/ml) for 48 h.

FACS analysis

Fluorescence-activated cell sorting (FACS) analysis was performed on a FACSCalibur (Becton Dickinson, San Jose, CA, USA) equipped with an argon gas laser. A standard band-pass filter was used to determine green fluorescence intensity. Approximately 5×10^4 cells from each sample were analyzed. The FL1 signal was acquired in logarithmic mode, and the data were analyzed with CellQuest software (Becton Dickinson).

RNA dot blot analysis

To estimate the retroviral supernatant titers, RNA dot blot analysis was performed as described previously [22]. Briefly, nylon membranes (Hybond N^+; Amersham Life Sciences, Arlington Heights, IL, USA) were soaked for 10 min in 1× SSC (0.15 M NaCl, 0.015 M sodium citrate) and placed onto a manifold dot blot apparatus (Schleicher & Schuell Inc., Keene, NH, USA). To avoid detection of the adenovirus DNA, supernatant (180 μl) without denaturing procedure was transferred onto nylon membranes using the manifold dot blot apparatus and vacuum suction. The membranes were cross-linked by UV and hybridized with a MMLV *psi* cDNA probe that had been randomly labeled with [α^{32}P] dCTP (Megaprime DNA labeling systems, Amersham Life Sciences). The membranes were subjected to phosphoimager analysis, and dot blot densities were measured with a Bio imaging analyzer (BAS1500; Fuji Photo Film Co., Ltd., Tokyo, Japan). Dot densities were expressed as relative pixel intensity and retroviral titers were determined by comparison with the known expression titer in a supernatant of the retrovirus vector LASN [23].

Adenovirus detection in the retroviral supernatants

To assay for the presence of biologically active adenovirus in the retroviral supernatants, 293 cells (1×10^4) were incubated with 200 μl of samples for 72 h. The presence of adenovirus was assessed by monitoring for evidence of cytopathic effects and the detachment of the cells 2 weeks later.

GCV sensitivity assay

The killing effect of GCV (Syntex, Palo Alto, CA, USA) was measured by ^{3}H-thymidine incorporation [24]. Briefly, 5×10^4 cells were cultured in 96-well flat-bottomed microtiter plates in the presence of varying concentrations (0–50 μM) of GCV. After 42 h the cells were pulsed with 0.25 μCi per well of ^{3}H-thymidine (Amersham International, Bucks, UK) and harvested 6 h later. The effect of GCV on tumor cell proliferation is expressed as a percentage of the thymidine incorporation found in the identical cultures not treated with GCV.

Molecular analysis

To confirm the integration of a retrovirus vector genome rescued by trans-complementation of the hybrid vectors, PCR analysis was conducted with high molecular weight DNA extracted from the target cells 14 days post-transduction (DNA extraction kit, Qiagen Inc.). A PCR-amplified DNA product (560 bp) was extended from the 5′ LTR or the 3′ LTR of the retroviral vector (sense primer 5′ AGGGCCAAGAACAGATGAGACAGC 3′) to a region downstream of the 5′ LTR (antisense primer: 5′ GTACAGACGCAGGCGCATAACATC 3′). Conversion of the 3′ LTR to the 5′ LTR after provirus integration was confirmed by digestion with *Xba* I. The PCR product was transferred to a nylon membrane (Hybond N^+; Amersham Life Sciences) by the capillary transfer method.

Vector treatment *in vivo*

Athymic female BALB/c *nu/nu* mice (Clea Japan, Tokyo, Japan) were inoculated subcutaneously with 9L cells (3×10^6 cells) in 100 μl Hank's balanced salt solution containing 25% (v/v) basement membrane matrix (Matrigel; BD Biosciences, Franklin Lakes, NJ, USA). The tumors were allowed to grow *in vivo* to an average volume of 70 mm^3 (tumor volumes were calculated as $a \times b^2 \times 0.5$ where a is the length and b is the width of the tumor in millimeters). The established subcutaneous tumors were injected with a control vector (group 1, AVC2.null), a therapeutic vector (group 2, AVC2.GCTK), a combination of vectors without the *gag-pol* expressing vector (group 3, AVC2.GCTK, AVC2.null, AxTetVSVG, and AV-rtTA), or the complete set of trans-complementing adenoviral vectors that rescues the retrovirus (group 4, AVC2.GCTK, AxTetGP, AxTetVSVG, and AV-rtTA). In each group, 1.0×10^9 plaque-forming units (pfu) of each virus were administered. Viruses were injected at four sites in each tumor with 100 μl of the virus solution in total. The animals received doxycycline as a 10 mg/ml solution in 5% sucrose via their water bottles for a period of 3 days starting 24 h after virus administration. When the tumor volume reached an average volume of 100 mm^3, the tumor-bearing animals were treated with an intraperitoneal injection of GCV at 30 mg/kg or phosphate-buffered saline (PBS) twice a day for 14 consecutive days. Tumors with an average volume of 100 mm^3 require more than 2.0×10^9 pfu of HSV-*tk* expression adenovirus vector for their complete

elimination [12]. Tumor growth was monitored two to three times a week by measuring two perpendicular tumor diameters using calipers. The total observation time was 2 months after the virus injection. Animals with tumors larger than 1 cm in diameter were euthanized. Tumor elimination that continued for more than 4 weeks was considered to indicate complete regression.

Determination of HSV-*tk* transgene copy number

To estimate the amplified copy number of the transgene caused by the production of retroviral progeny, PCR analysis was conducted on high molecular weight DNA extracted from tumors (DNA extraction kit, Qiagen Inc.). Using pAVS6TK [12] as a standard, an HSV-*tk*-specific nucleotide sequence (nucleotides +418 to +737) was amplified by PCR. Primers were chosen with the assistance of the computer program Primer Express (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). We performed BLAST searches against dbEST and nr (the non-redundant set of GenBank, EMBL, and DDBJ database sequences) to confirm the total gene specificity of the nucleotide sequences chosen as primers. The specificity of the primer sets was confirmed by sequencing the PCR amplification products cloned into the pGEM-T vector (Promega, Madison, WI, USA). Quantitative values were obtained from the threshold cycle (*Ct*) number that indicated exponential amplification of the PCR product (ABI PRISM 7700 sequence detection system; Applied Biosystems, Foster City, CA, USA). To normalize each sample, we also quantified the copy number of the *GAPDH* gene. The relative target gene copy number was also normalized with a calibrator (tumors treated with AVC2GCTK alone). The final result, expressed as *N*-fold differences in target gene copy number relative to the GAPDH gene and the calibrator, was determined by the following formula: $N\text{target} = 2^{\text{corrected}\Delta Ct(\text{GAPDH}-\text{TK})}$. *Ct* values of the sample were determined by subtracting the average *Ct* value of the target gene from the average *Ct* value of the *GAPDH* gene.

In situ hybridization

Tumors from mice subjected to intratumoral injection with adenovirus along with intraperitoneal PBS treatment were removed 14 days after the injection and *in situ* hybridization was performed using an antisense RNA probe specific for the HSV-*tk* transcripts. Tissue specimens from subcutaneous tumors were fixed for 12 h in 4% paraformaldehyde at 4 °C, cut into 30-μm sections and mounted on APS-coated slides. Using pAVS6TK [12] as a template, an HSV-*tk*-specific nucleotide sequence (nucleotides +418 to +737) was amplified by PCR and cloned into pGEM-T vector (Promega). The HSV-*tk* RNA probes were synthesized and labeled with digoxigenin (DIG)-labeled deoxyuridine triphosphate by using a DIG RNA-labeling kit (F. Hoffmann-La Roche Ltd, Basel, Switzerland) according to the manufacturer's instructions. A DIG-labeled antisense RNA probe was obtained by using DIG RNA labeling mixture with a *Spe* I-cut linearized template and T7 RNA polymerase. Similarly, a sense probe was prepared for negative control experiments by using an *Nco* I-digested template and SP6 RNA polymerase with the DIG RNA labeling mixture.

The 30-μm tumor sections were pretreated with 100 μg/ml proteinase K at 37 °C for 20 min. These sections were rinsed with 2 mg/ml glycine and then incubated with hybridization buffer containing 100 ng of labeled RNA probe in a moist chamber at 42 °C overnight. After hybridization, the sections were washed in 1 × SSC for 10 min at room temperature, 0.2 × SSC for 20 min at 42 °C, 2 × SSC for 2 min at room temperature, and digested with 10 μg/ml RNase A at 37 °C for 20 min. The sections were incubated in 0.5% blocking reagent for 60 min and then in a 1 : 100 dilution of alkaline phosphatase conjugated anti-DIG antibody for 60 min in a moist chamber, followed by rinsing with Tris-buffered saline. The alkaline phosphatase reaction was visualized with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium. The sections were counterstained with methylgreen and coverslipped for light microscopy.

Results

Adeno-retroviral hybrid vectors can rescue an integrated retroviral genome from human glioma cells

To determine if an integrated retroviral genome could be rescued from glioma cells by use of our adeno-retroviral hybrid system, a human glioma cell line harboring a retroviral vector expressing GFP (D54GFPneo) was infected with a combination of adenoviral vectors that express proteins required for retroviral packaging proteins. Two trans-complementing adeno-retroviruses were used. AxTetGP expresses the MMLV gag-pol and AxTetVSVG expresses the VSV-G, which acts as the retrovirus envelope protein. In addition, a third adenovirus (AV-rtTA) was employed to regulate the expression of these viruses. As a control system in which retrovirus production does not occur, the AxTetGP virus was replaced by the non-expressing adenovirus vector AVC2null. Cells were infected with the appropriate adenovirus vectors for 3 h at various multiplicities of infection (MOIs). Free adenovirus was removed by washing cells with medium supplemented with human γ-globulin. Putative retrovirus-containing supernatants were harvested 48 h post-adenoviral transduction and used to transduce rat glioma 9L cells (Figure 2). Analysis of the 9L cells showed significant levels of GFP 2 days after the initiation of transduction (Figure 2A). In contrast, target 9L cells exposed to supernatants obtained from D54GFPneo cells transduced with the null adenoviral

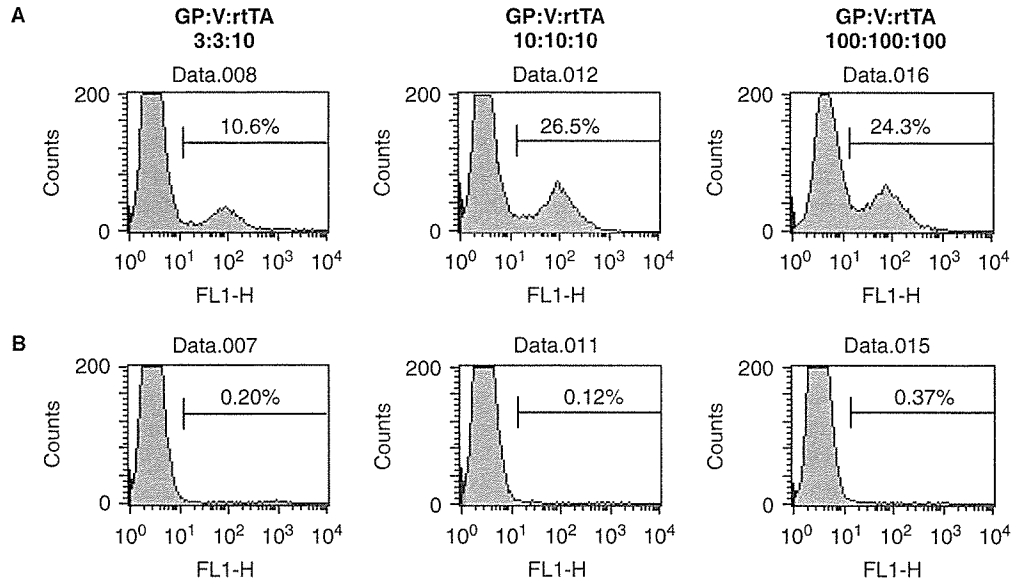

Figure 2. Rescue of an integrated GFP-expressing retroviral vector in 9L glioma cells by transduction with trans-complementing adeno-retroviral hybrid vectors. Putative retrovirus-containing supernatants were obtained from a human glioma cell line carrying an integrated retrovirus (D54GFPneo) following transduction with trans-complementing adeno-retroviral hybrid vectors at various MOIs. The putative retroviral supernatants were used to transduce 9L glioma cells so that the production of progeny retroviruses carrying the integrated GFP gene could be detected. (A) FACS analysis of 9L cells exposed to supernatants from D54GFPneo cells that had been transduced with all of the adeno-retroviral vectors required for the generation of a retrovirus, namely, AxTetGP (GP), AxTetVSVG (V), and AV-rtTA (rtTA). (B) FACS analysis of 9L cells exposed to supernatants from D54GFPneo cells transduced with all of the adeno-retroviral vectors except for the retroviral gag-pol-expressing vector, which was replaced by the control AVC2.null vector

vector plus the adenoviral vectors expressing the VSV-G envelope protein and the rtTA showed little or no GFP expression (Figure 2B). The percentage of target cells that were transduced reached a plateau when an MOI of 10 was used for each of the input adenoviruses. No biologically active adenovirus was detected in any of the supernatants.

In situ transduction efficiency of the adeno-retroviral hybrid vector system in glioma cells

To assess the effect of the *in situ* production of retroviral vector on the transduction efficiency of glioma cells, 9L cells were transduced with adeno-retroviral hybrid vectors expressing a retroviral genome (AVC2.GCEGFP) together with all of the retroviral packaging proteins (AxTetGP, AxTetVSVG, and AV-rtTA). Four days after the adenoviral transduction, a higher percentage of the cells were GFP-positive when they had been exposed to all of the trans-complementing adeno-retroviral vectors compared with cells exposed to AVC2.GCEGFP alone or when AxTetGP was replaced by the non-expressing adenoviral vector AVC2.null (Figure 3A). To confirm the long-term transgene expression from the retrovirus progeny generated by the adeno-retroviral vectors, we compared 9L cells transduced by all of the required adeno-retroviral hybrid viruses with those transduced by the control vector combination (AVC2.GCEGFP, AVC2.null, AxTetVSVG, and AV-rtTA). These cells were mixed with non-infected cells at a ratio of 10% transduced cells to 90% non-transduced cells and passaged every 7 days. Two weeks after the transduction, GFP reporter gene persistence and expression were analyzed by fluorescent microscopy. The GFP-positive cells from the group infected with AVC2.GCEGFP, AxTetGP, AxTetVSVG, and AV-rtTA were present in a clustered outgrowth after long-term culture, suggesting local retroviral spread and clonal origin (data not shown). This was in contrast to the cells infected with the control combination of adenoviruses (AVC2.GCEGFP, AVC2null, AxTetVSVG, and AV-rtTA), which showed attenuated GFP expression.

To determine how long cells can produce retrovirus after being infected with the vectors, a time course experiment was performed. The 9L cells were infected with all of the required adeno-retroviral hybrid viruses (AVC2.EGFP, AxTetGP, AxTetVSVG, and AV-rtTA) at an MOI of 3 in the presence of doxycycline. The titers increased and reached nearly 4×10^5 TU/ml by day 2, were maintained at similar levels until day 4, and then decreased (Figure 3B). At day 6, retrovirus production was still observed at a titer of 9×10^4 TU/ml. Thus, infected 9L cells continued to sustain retrovirus production for at least 6 days post-infection. The retention of transgene expression over time in infected cells was determined by evaluating the percentage of EGFP-positive cells by FACS analysis. The percentage of EGFP-positive cells increased over time, while the control group reached plateau at day 4 (Figure 3C).

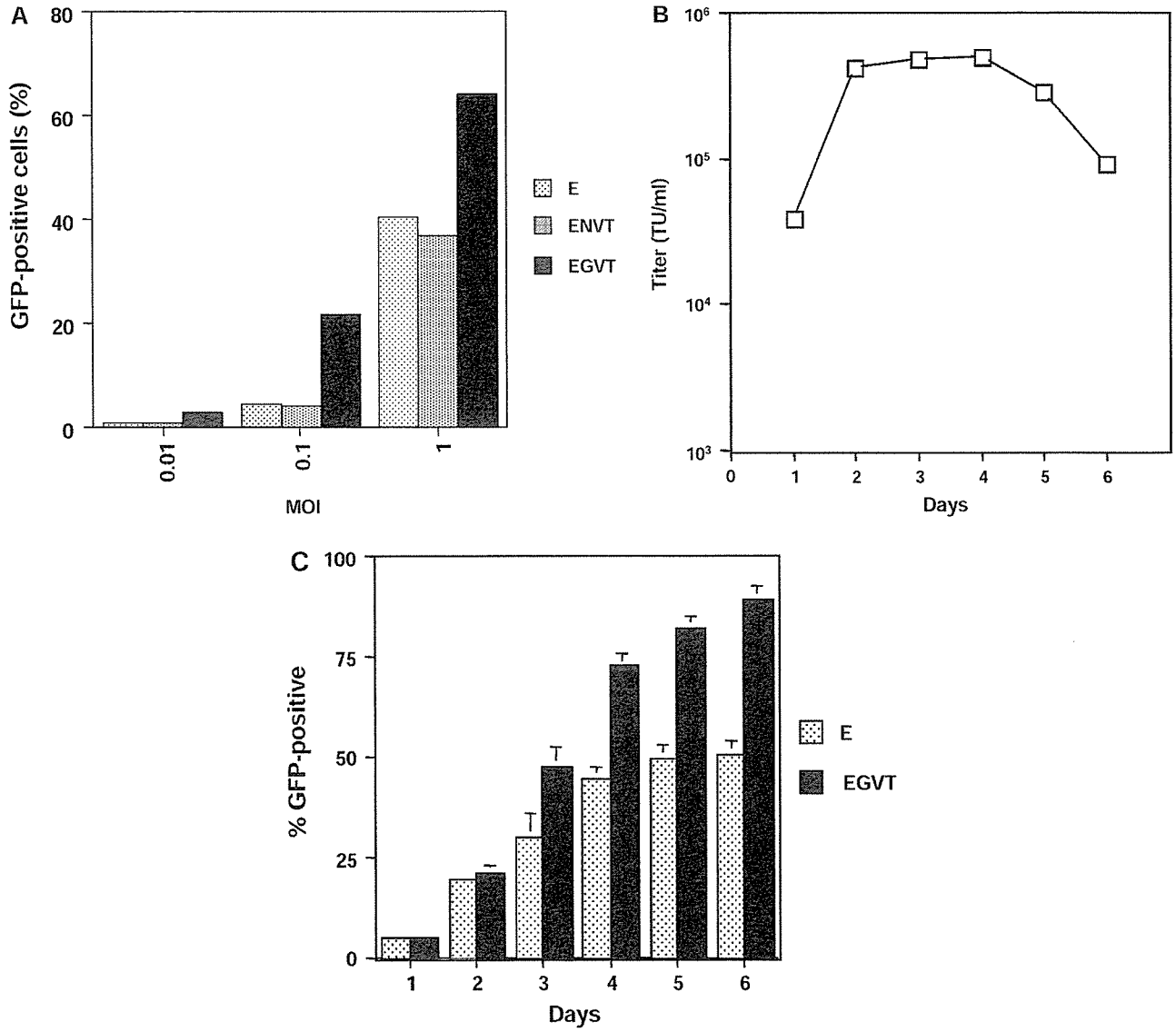

Figure 3. Transduction efficiency of the adeno-retroviral hybrid vector system in 9L cells. (A) Rat 9L glioma cells were infected with the trans-complementing adeno-retroviral vectors at a variety of MOIs (0.01, 0.1, and 1 of each virus) in the presence of 1 μg/ml doxycycline. The hybrid adeno-retroviruses used were as follows: AVC2.GCEGFP alone (E), AVC2.GCEGFP, AVC2null, AxTetVSVG, and AV-rtTA (ENVT), or AVC2.GCEGFP, AxTetGP, AxTetVSVG, and AV-rtTA (EGVT). Cells were harvested for FACS analysis of GFP expression 4 days after transduction. (B) Time course of retroviral production by 9L cells infected with the hybrid vectors. Cells were infected with AVC2.GCEGFP, AxTetGP, AxTetVSVG, and AV-rtTA at an MOI of 3 of each virus in the presence of doxycycline. At different time points, the medium was replaced and the supernatant was titrated for EGFP expression on 293 cells. Data shown represent means from three independent experiments. (C) The percentage of EGFP-positive cells transduced with the hybrid vectors was determined at various time points by FACS. Cells were infected with AVC2.GCEGFP (E) or with AVC2.GCEGFP, AxTetGP, AxTetVSVG, and AV-rtTA (EGVT) at an MOI of 3 of each virus in the presence of doxycycline. Data shown represent means and standard deviations from three independent experiments

Characterization of an adeno-retroviral vector carrying the HSV-*tk* gene

To demonstrate that the retroviral vector cassette within AVC2.GCTK could be used as a retroviral genome template, the retroviral producer cell line FLYA13 was transduced with the adeno-retroviral hybrid vector AVC2.GCTK at an MOI of 10. The retroviral supernatants harvested 48 h post-transduction gave a titer of 1×10^7 TU/ml, which is consistent with titers obtained using this cell line for the conventional production of retrovirus [5].

To examine the generation of the progeny retroviruses in glioma cells using this AVC2.GCTK adeno-retroviral vector system, we transduced rat glioma 9L cells with this vector and the trans-complementing adenovirus carrying the retroviral gag-pol and VSV-G proteins at an MOI of 10 for each, followed by doxycycline treatment. The retroviral titer of supernatants harvested 4 days after transduction, as measured by RNA dot blot analysis, was estimated to be 4×10^5 TU/ml. This experiment was performed several times using different pools of supernatants and produced similar results in all cases.

The HSV-*tk* adeno-retrovirus enhances GCV pro-drug killing of glioma cells

To assess if the killing effect of HSV-*tk* and GCV is enhanced as a result of the use of our adeno-retroviral hybrid vector system, a GCV-sensitivity assay was performed using a mixture of transduced and non-transduced 9L cells at a ratio of 1:19 or 1:9. Three different groups of transduced 9L cells were studied: (1) cells transduced with AVC2.null alone (MOI 40); (2) cells transduced with AVC2.GCTK, AVC2.null, AxTetVSVG, and AV-rtTA (MOI of 10 each); and (3) cells transduced with AVC2.GCTK, AxTetGP, AxTetVSVG, and AV-rtTA) (MOI of 10 each). The cells were analyzed for GCV sensitivity 48 h post-transduction. A dose range of GCV was used (0.01–100 μM). Representative data are shown in Figure 4. In the presence of GCV, infected cells mixed with non-infected cells at a ratio of 5% to 95% that were transduced with all the adeno-retroviral vectors except that AxTetGP, which is required for the generation of retrovirus, had an IC50 value of 46.8 μM (Figure 4A). In contrast, 9L transduced with the progeny-producing combination of adeno-retroviruses had an IC50 value of 1.1 μM. The increase in sensitivity to GCV that the ability to produce progeny conferred to the 9L glioma cells is thus more than one log. Similar results were obtained when infected cells were mixed with non-infected cells at a ratio of 10% to 90% (Figure 4B).

Proviral integration

The generation of an integrated HSV-*tk* provirus by trans-complementation of the adeno-retroviral vectors was verified by analysis of the flanking retroviral LTR sequences. Typical retrovirus reverse transcription and integration resulted in the re-duplication of the 3′ LTR of the retrovirus to form the 5′ LTR (Figure 5A). The 5′ and 3′ LTR sequences of the retroviral vector in the original adeno-retroviral vector differed slightly as the 5′ LTR was derived from MMLV and the 3′ LTR was from MPSV. Re-duplication of the 3′ LTR as the result of retroviral transduction, reverse transcription, and integration should result in the generation of two LTRs with the same sequence. To assess if the expected re-duplication of the 3′ LTR occurred following the transduction of 9L cells with the trans-complementing adenoviruses and the AVC2.GCTK adeno-retroviral vector, we extracted high molecular weight cellular DNA from the cells transduced with AVC2.GCTK, AxTetGP, AxTetVSVG, and AV-rtTA. A PCR product of the expected size was amplified from the DNA sequences within the LTR to a region downstream of 5′ LTR. *Xba* I digestion of the PCR product resulted in the generation of fragments of the expected size, which suggests that the 3′ LTR had been converted into the 5′ LTR following the generation of progeny retrovirus (Figure 5B).

Therapeutic effect of the HSV-*tk* adeno-retroviral vector system *in vivo*

To assess if intratumoral administration of the trans-complementing HSV-*tk* adeno-retroviral vectors leads to the *in situ* production of a retrovirus expressing HSV-*tk* and thus enhanced sensitivity to GCV killing, 9L tumors established in *nu/nu* mice were injected with either AVC2.null alone (n = 20, group 1), AVC2.GCTK alone (n = 20, group 2), AVC2.GCTK, AVC2.null, AxTetVSVG, and AV-rtTA (n = 20, group 3), or AVC2.GCTK, AxTetGP, AxTetVSVG, and AV-rtTA (n = 16, group 4). Subsequently, half the animals in each group (n = 10 or 8 in each group) received intraperitoneal injections of either PBS or GCV. Figure 6 shows the tumor size in *nu/nu* mice after vector administration. Compared with treatment with combinations that are insufficient for retrovirus production, treatment with the ideal combination of vectors significantly inhibited the growth of the established tumors (group 4 vs. group 3 plus GCV, $p < 0.05$ by the

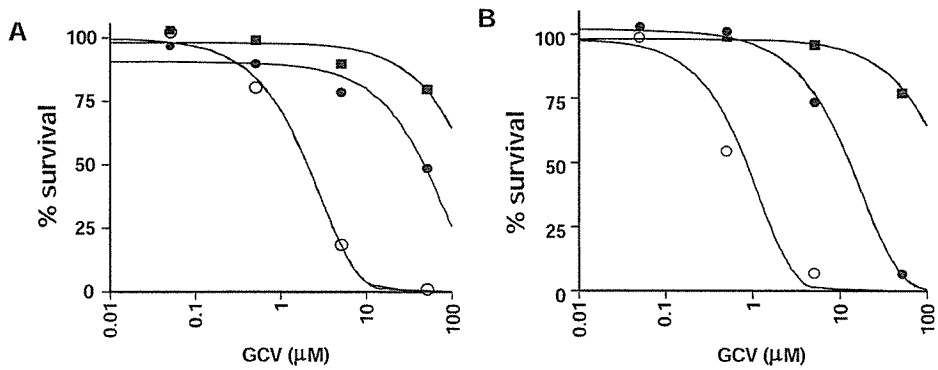

Figure 4. Enhanced killing of rat 9L glioma cells following transduction with adeno-retroviral vectors. GCV-sensitivity assays were performed using mixtures of transduced and non-transduced 9L cells at a ratio of 1:19 (A) or 1:9 (B). Three different groups of transduced cells were used, namely, (1) AVC2.null at an MOI of 40 (■); (2) AVC2.GCTK, AVC2null, AxTetVSVG, and AV-rtTA (MOI of 10 for each) (●); and (3) AVC2.GCTK, AxTetGP, AxTetVSVG, and AV-rtTA (MOI of 10 for each) (○). The transduced 9L cells were harvested 48 h post-transduction and the killing effect of GCV was measured by ^{3}H-thymidine incorporation. The effect on tumor cell proliferation is expressed as a percentage of the thymidine incorporation found in identical cultures that had not been treated with GCV

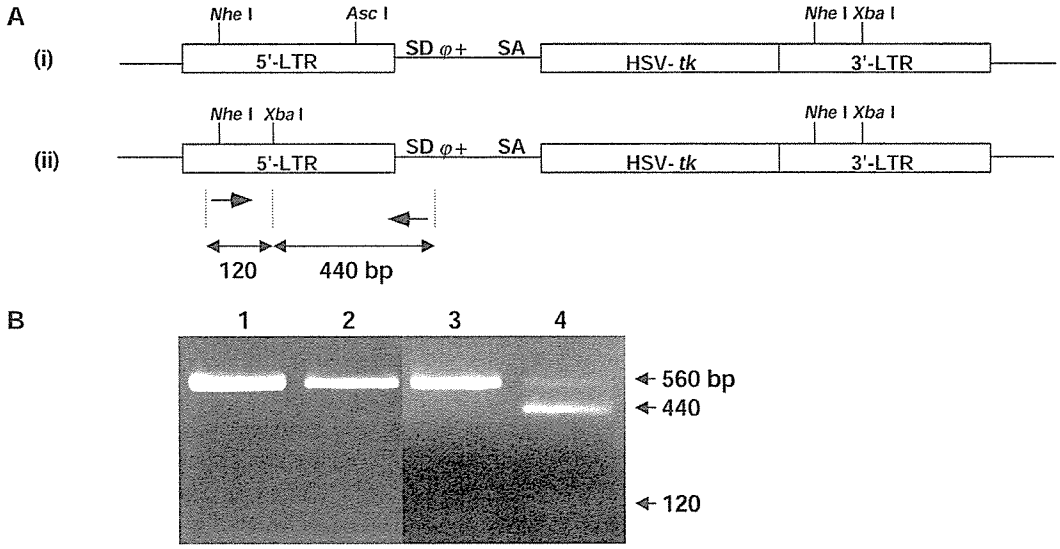

Figure 5. Integration of progeny retrovirus generated from the HSV-*tk* adeno-retroviral vectors. Genomic DNA of subcutaneous 9L tumors transduced with AVC2.GCTK, AxTetGP, AxTetVSVG, and AV-rtTA was extracted for PCR analysis to determine if there had been successful integration of the progeny retroviruses. (A) (i) Schematic representation of the retroviral vector incorporated in the adeno-retroviral vector AVC2-GCTK. The 3′ LTR of AVC2.GCTK contains a unique *Xba* I site. (ii) Re-duplication of the 3′ LTR into the 5′ LTR during retroviral replication and proviral integration generates an *Xba* I site within the PCR product. (B) PCR products amplified from AVC2.GCTK DNA (lanes 1 and 2) and high molecular weight DNA purified from 9L cells transduced with the hybrid trans-complementing adeno-retroviruses (lanes 3 and 4). The PCR products in lanes 2 and 4 were digested to completion with *Xba* I

Student's t test). Since groups 3 and 4 contain the VSV-G expression vector, VSV-G-related toxicity might induce different initial growth rates. Intratumoral injection of all the trans-complementing HSV-*tk* adeno-retroviral vectors followed by GCV treatment resulted in complete tumor regression in four of the eight tumors that lasted longer than 4 weeks, while none of the tumors in control animals were eliminated ($p < 0.05$ by the Fisher's exact probability test). Similar results were obtained in a repeat of this experiment.

Enhanced expression of HSV-*tk* in tumors accompanying the generation of retroviral progeny

To determine if the enhanced killing seen following intratumoral injection of the trans-complementing HSV-*tk* adeno-retrovirus was due to the *in situ* generation of HSV-*tk* expressing retrovirus, we estimated the relative copy number of the HSV-*tk* transgene present in the high molecular weight DNA extracted from the tumors of the mice treated as described above (Table 1). The *GAPDH* gene was used as a reference gene to correct for variation in the amounts of DNA. The relative copy number of the HSV-*tk* gene in tumors lacking retroviral production (group 3, AVC2.GCTK, AVC2.null, AxTetVSVG, and AV-rtTA) was similar to that in the control treatment (group 2, AVC2.GCTK), whereas, compared with group 2, it was 89- and 258-fold higher in tumors receiving AVC2.GCTK, AxTetGP, AxTetVSVG, and AV-rtTA (group 4).

To determine the expression pattern of the HSV-*tk* transcripts within a tumor, *in situ* hybridization analysis was performed using an RNA probe specific for HSV-*tk*. The HSV-*tk* transcripts in the tumors that did not produce

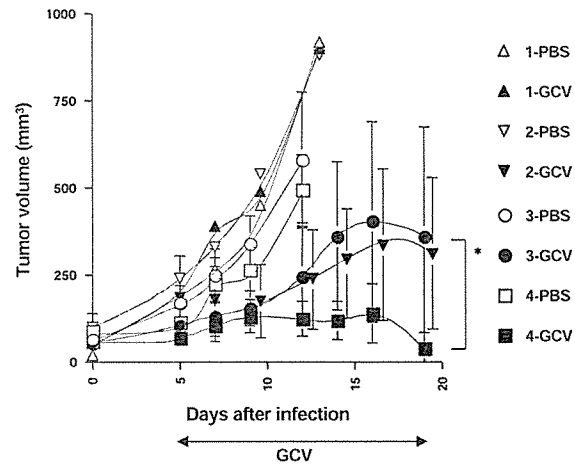

Figure 6. Effects of the hybrid vector system on xenograft growth in athymic mice. To establish subcutaneous tumors, 9L cells were inoculated subcutaneously into BALB/c *nu/nu* mice. Eight days after the inoculation, the established subcutaneous tumors were injected with either the control vector (1, AVC2.null, n = 20), the therapeutic vector (2, AVC2.GCTK, n = 20), a combination of vectors lacking AxTetGP (3, AVC2.GCTK, AxTetVSVG, and AV-rtTA; n = 20), or the complete set of vectors (4, AVC2.GCTK, AxTetGP, AxTetVSVG, and AV-rtTA; n = 16). Animals received doxycycline as a 10 mg/ml solution with 5% sucrose via their water bottles for a period of 3 days starting at 24 h after the injection. When the tumor volume reached an average volume of 100 mm^3, half the animals in each group were treated with intraperitoneal injections of GCV at 30 mg/kg or PBS twice a day for 14 consecutive days. Each point represents the average volume of the tumors ± one standard deviation. *$p < 0.05$ by the Student's *t* test. Similar results were obtained in a repeat of this experiment

Table 1. Relative HSV-*tk* transgene copy number in tumors treated with adeno-retroviral trans-complementing viruses as measured by real-time PCR. The relative copy number of the HSV-*tk* gene was determined as the ratio of the copy numbers in tumors treated with AVC2.GCTK alone to the copy numbers in the other treatment group. The copy number of the reference gene *GAPDH* was also determined to correct for variation in the amounts of DNA. The final results are expressed as *N*-fold differences in the HSV-*tk* gene copy number relative to the GAPDH gene

	$2^{\text{corrected}\Delta Ct(GAPDH-TK)}$	
	Expt 1	Expt 2
vector	<0.05	<0.05
AVC2GCTK	1.0	1.0
AVC2GCTK, VSV-G	1.3	3.1
AVC2GCTK, VSV-G, gag-pol	89.3	258

retroviral progeny were restricted to a relatively small area (Figure 7A). In contrast, tumors that had received all of the trans-complementing adeno-retroviruses showed evidence of higher levels and a wider dispersion of the HSV-*tk* RNA signal (Figure 7C). Controls using a sense probe showed no specific reactivity (Figures 7B and 7D).

Discussion

In this study, we sought to engineer adeno-retroviral hybrid vectors that would produce, *in situ* in tumor cells, progeny retrovirus particles that express the therapeutic HSV-*tk* gene. The aim of this system was to improve the distribution of the therapeutic gene, which in turn should reduce the amount of GCV required to kill the transduced cells. We show that a single-step transduction of tumor cells with trans-complementing hybrid adeno-retroviral vectors effectively turns these cells into retrovirus vector-producing cells, which in turn facilitates the transduction of adjacent cells. This significantly increases the expression of the transgene. Moreover, we showed that as a result of the pseudotyped retrovirus production following trans-complementation of the adeno-retroviral hybrid viruses, the transduction efficiency both *in vitro* and *in vivo* was enhanced. In addition, we found that the trans-complementing adeno-retroviral hybrid vector system expressing HSV-*tk* and producing progeny retroviruses reduced by one log the amount of GCV required to induce significant *in vitro* killing of 9L glioma cells. Treatment with this system also completely inhibited the growth of established

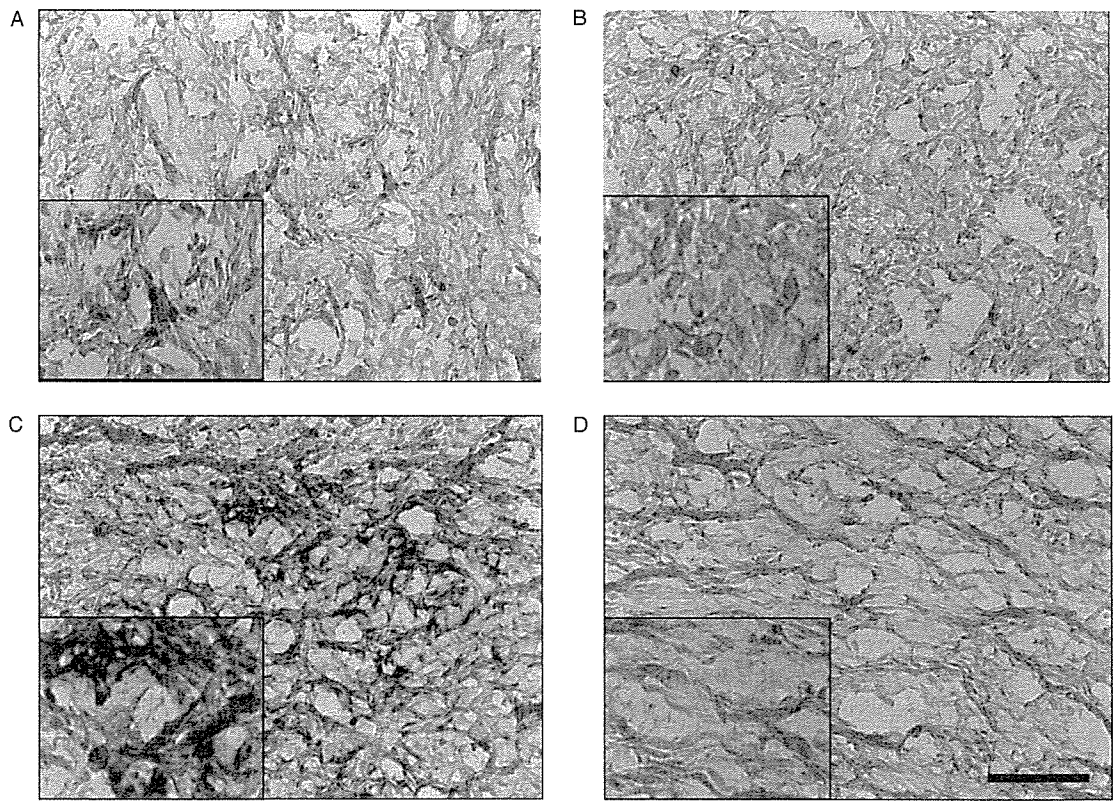

Figure 7. Enhanced expression of HSV-*tk* transcripts in 9L xenografts in athymic mice. Tumors from mice subjected to intratumoral injection with adenovirus along with PBS treatment were removed 14 days after the injection and *in situ* hybridization was performed using an antisense RNA probe specific for the HSV-*tk* transcripts (A and C). In tumor sections from mice treated with AVC2.GCTK, AxTetVSVG, and AV-rtTA (A), HSV-*tk* was rarely detectable. However, in tumor sections from mice treated with the complete set of vectors (AVC2.GCTK, AxTetGP, AxTetVSVG, and AV-rtTA), the HSV-*tk* RNA signal was more intense and shows a wider pattern of dispersion through the tumor section (C). *In situ* hybridization for HSV-*tk* using the anti-sense probe (A and C) or the sense probe (B and D). Results from one of several independent experiments are shown here. Similar results were obtained in the other experiments. Magnification, ×100. Scale bar, 100 μm. Insert, 2× overview

subcutaneous tumors in nude mice, and we showed that this was probably due to the amplification of the HSV-*tk* gene copy number and the consequent enhanced spread of the HSV-*tk* product throughout the tumor.

We and others have previously described hybrid vector systems that use adenoviral vectors to deliver retroviral vector and packaging proteins into cells [3–9]. These systems benefit from the efficient gene transfer characteristics of adenoviral vectors along with the stable and long-term gene expression that is typical of retroviral vectors. Initial studies of the co-transduction of adeno-retroviral hybrid vectors showed that transient retrovirus-producing cells can be successfully generated and that these could subsequently cause the transduction of neighboring cells [3,7]. Torrent *et al.* [9] also reported a study with a chimeric vector system that resulted in 10- to 50-fold transgene amplification *in vivo*, although they did not examine the distribution of the transgene or the therapeutic efficacy of their system. In the study reported here, we have altered these hybrid adeno-retroviral vector systems so that the tumor-killing effect of adenovirus-mediated HSV-*tk*/GCV therapy *in situ* is enhanced. Compared with the adenovirus delivery systems that lack retrovirus production, our system provides 89- and 258-fold transgene amplification *in situ* and stronger and wider dispersion of the transgene RNA signal. The enhanced amplification efficiency is most likely due to the *in situ* generated progeny retrovirus particles bearing the VSV-G envelope protein, which has a broader host range and higher transduction efficiency than murine leukemia virus derived retrovirus [7]. Murine leukemia virus derived retroviral vectors have had limited application in gene therapy because of low transduction efficiency of tissues, both *in vitro* and *in vivo*. One study showed that the transduction efficiency of the amphotropic vector into human cancer cells was not dose-dependent and reached a plateau or even decreased, especially at high MOIs [25]. This may be attributed in part to the presence of the envelope protein and non-infectious particles that compete for the receptor of infectious amphotropic viruses. In contrast, the receptor for VSV-G exists in abundance on the cell surface. Thus, our hybrid vector system pseudotyped with the VSV-G envelope glycoprotein is an alternative tool for efficient transduction.

For safety reasons, the retroviral functions in our system were split into different adenoviral backbones. However, there are some reports of studies that use a single helper-dependent adenovirus (HDAd) vector to accommodate large inserts. A vector system that uses an HDAd vector as a carrier to deliver a replication-competent ecotropic retrovirus vector has been developed [26]. An adenovirus/lentivirus hybrid vector on a single HDAd backbone was also developed for stable integration [27]. Although adenovirus-mediated transduction seems to be efficient in most cancers, there are several reports of studies using different viral components that convert tumor cells into retroviral producer cells. One of these employs a single-step system with herpes simplex virus/Epstein-Barr virus hybrid amplicons that converts cells into retrovirus vector producers [28]. Infection of primary gliomas with this system resulted in the production of retrovirus vectors and long-term retention *in vitro*. Poxviral/retroviral chimeric vectors also allow cytoplasmic production of defective retroviral particles [29]. Vaccinia-mediated expression of retroviral vector particles could be observed as early as 3 h post-infection and resulted in the stable transduction of NIH/3T3 cells. An alphavirus/retrovirus hybrid vector has also been used to transduce the retroviral vector packaging cell line [30]. The produced factor IX minigene-containing retroviral vectors were used for stable transduction *in vitro*.

When we used our hybrid vector system to treat a subcutaneous tumor model, we detected enhanced transgene expression together with an increased therapeutic effect *in vivo*. This suggests that the retroviral progeny are efficiently produced *in situ*. Although the efficacy of this system has to be evaluated in orthotopic models, current observations suggest that it is a promising method that has many possible applications in cancer gene therapy.

Acknowledgements

We wish to thank Dr. Tom Shenk for providing dl327 and Drs. Hiroshi Amanuma and Kiyotake Tobita for their support. We also wish to thank Ms. Miyoko Mitsu and Dr. Masahide Koremoto for their technical support and encouragement. This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, by the Jichi Medical School young investigator award, by the Uehara Memorial Foundation, by the Osaka Cancer Research Foundation, and by the Nakajima Foundation.

References

1. Ram Z, Culver KW, Oshiro EM, *et al.* Therapy of malignant brain tumors by intratumoral implantation of retroviral vector-producing cells. *Nat Med* 1997; **3**: 1354–1361.
2. Rainov NG. A phase III clinical evaluation of herpes simplex virus type 1 thymidine kinase and ganciclovir gene therapy as an adjuvant to surgical resection and radiation in adults with previously untreated glioblastoma multiforme. *Hum Gene Ther* 2000; **11**: 2389–2401.
3. Feng M, Jackson WH Jr, Goldman CK, *et al.* Stable in vivo gene transduction via a novel adenoviral/retroviral chimeric vector. *Nat Biotechnol* 1997; **15**: 866–870.
4. Bilbao G, Feng M, Rancourt C, Jackson WJ, Curiel DT. Adenoviral/retroviral vector chimeras: a novel strategy to achieve high-efficiency stable transduction in vivo. *FASEB J* 1997; **11**: 624–634.
5. Ramsey WJ, Caplen NJ, Li Q, Higginbotham JN, Shah M, Blaese RM. Adenovirus vectors as transcomplementing templates for the production of replication defective retroviral vectors. *Biochem Biophys Res Commun* 1998; **246**: 912–919.
6. Lin X. Construction of new retroviral producer cells from adenoviral and retroviral vectors. *Gene Ther* 1998; **5**: 1251–1258.
7. Caplen NJ, Higginbotham JN, Scheel JR, *et al.* Adeno-retroviral chimeric viruses as in vivo transducing agents. *Gene Ther* 1999; **6**: 454–459.
8. Duisit G, Salvetti A, Moullier P, Cosset FL. Functional characterization of adenoviral/retroviral chimeric vectors and their use for efficient screening of retroviral producer cell lines. *Hum Gene Ther* 1999; **10**: 189–200.

9. Torrent C, Jullien C, Klatzmann D, Perricaudet M, Yeh P. Transgene amplification and persistence after delivery of retroviral vector and packaging functions with E1/E4-deleted adenoviruses. *Cancer Gene Ther* 2000; **7**: 1135–1144.
10. Okada T, Ramsey J, Munir J, Wildner O, Blaese M. Efficient directional cloning of recombinant adenovirus vectors using DNA-protein complex. *Nucleic Acids Res.* 1998; **26**: 1947–1950.
11. Treisman J, Hwu P, Minamoto S, *et al.* Interleukin-2-transduced lymphocytes grow in an autocrine fashion and remain responsive to antigen. *Blood* 1995; **85**: 139–145.
12. Okada T, Shah M, Higginbotham JN, *et al.* AV.TK-mediated killing of subcutaneous tumors in situ results in effective immunization against established secondary intracranial tumor deposits. *Gene Ther* 2001; **8**: 1315–1322.
13. Stein R, Ziff EB. HeLa cell beta-tubulin gene transcription is stimulated by adenovirus 5 in parallel with viral early genes by an E1a-dependent mechanism. *Mol Cell Biol* 1984; **4**: 2792–2801.
14. Graham FL, Prevec L. Adenovirus-based expression vectors and recombinant vaccines. *Biotechnology* 1992; **20**: 363–390.
15. Yoshida Y, Emi N, Hamada H. VSV-G-pseudotyped retroviral packaging through adenovirus-mediated inducible gene expression. *Biochem Biophys Res Commun* 1997; **232**: 379–382.
16. Cosset FL, Takeuchi Y, Battini JL, Weiss RA, Collins MK. High-titer packaging cells producing recombinant retroviruses resistant to human serum. *J Virol* 1995; **69**: 7430–7436.
17. Benda P, Someda K, Messer J, Sweet WH. Morphological and immunochemical studies of rat glial tumors and clonal strains propagated in culture. *J Neurosurg* 1971; **34**: 310–323.
18. Bethea JR, Gillespie GY, Chung IY, Benveniste EN. Tumor necrosis factor production and receptor expression by a human malignant glioma cell line, D54-MG. *J Neuroimmunol* 1990; **30**: 1–13.
19. Graham FL, Smiley J, Russell WC, Nairn R. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J Gen Virol* 1977; **36**: 59–74.
20. Wildner O, Candotti F, Krecko EG, Xanthopoulos KG, Ramsey WJ, Blaese RM. Generation of a conditionally neo(r)-containing retroviral producer cell line: effects of neo(r) on retroviral titer and transgene expression. *Gene Ther* 1998; **5**: 684–691.
21. Wigler M, Pellicer A, Silverstein S, Axel R. Biochemical transfer of single-copy eucaryotic genes using total cellular DNA as donor. *Cell* 1978; **14**: 725–731.
22. Onodera M, Yachie A, Nelson DM, Welchlin H, Morgan RA, Blaese RM. A simple and reliable method for screening retroviral producer clones without selectable markers. *Hum Gene Ther* 1997; **8**: 1189–1194.
23. Onodera M, Nelson DM, Yachie A, *et al.* Development of improved adenosine deaminase retroviral vectors. *J Virol* 1998; **72**: 1769–1774.
24. Harstrick A, Casper J, Guba R, Wilke H, Poliwoda H, Schmoll HJ. Comparison of the antitumor activity of cisplatin, carboplatin, and iproplatin against established human testicular cancer cell lines in vivo and in vitro. *Cancer* 1989; **63**: 1079–1083.
25. Arai T, Takada M, Ui M, Iba H. Dose-dependent transduction of vesicular stomatitis virus G protein-pseudotyped retrovirus vector into human solid tumor cell lines and murine fibroblasts. *Virology* 1999; **260**: 109–115.
26. Soifer H, Higo C, Logg CR, *et al.* A novel, helper-dependent, adenovirus-retrovirus hybrid vector: stable transduction by a two-stage mechanism. *Mol Ther* 2002; **5**: 599–608.
27. Kubo S, Mitani K. A new hybrid system capable of efficient lentiviral vector production and stable gene transfer mediated by a single helper-dependent adenoviral vector. *J Virol* 2003; **77**: 2964–2971.
28. Sena-Esteves M, Saeki Y, Camp SM, Chiocca EA, Breakefield XO. Single-step conversion of cells to retrovirus vector producers with herpes simplex virus-Epstein-Barr virus hybrid amplicons. *J Virol* 1999; **73**: 10 426–10 439.
29. Holzer GW, Mayrhofer JA, Gritschenberger W, Dorner F, Falkner FG. Poxviral/retroviral chimeric vectors allow cytoplasmic production of transducing defective retroviral particles. *Virology* 1999; **253**: 107–114.
30. Wahlfors JJ, Morgan RA. Production of minigene-containing retroviral vectors using an alphavirus/retrovirus hybrid vector system. *Hum Gene Ther* 1999; **10**: 1197–1206.

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RESEARCH ARTICLE

Expression of human coagulation factor VIII in adipocytes transduced with the simian immunodeficiency virus agmTYO1-based vector for hemophilia A gene therapy

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We demonstrate that transduction of adipocytes with a simian immunodeficiency virus agm TYO1 (SIVagm)-based lentiviral vector carrying the human coagulation factor VIII gene (SIVhFVIII) resulted in expression of the human FVIII transgene in vitro and in db/db mice in vivo. Cultured human adipocytes were transduced with the SIVagm vector carrying the GFP gene in a dose-dependent manner and transduction of adipocytes with SIVhFVIII resulted in efficient expression of human coagulation factor VIII (hFVIII; 320±39.8 ng/10⁶ adipocytes/24 h) in vitro. Based upon successful transduction of adipocytes by SIV vectors carrying the lacZ gene in vivo in mice, the adipose tissue of db/db *mice was transduced with SIVhFVIII. There was a transient appearance of human FVIII in mouse plasma (maximum 1.8 ng/ml) on day 11 after the injection. Transcripts of human FVIII transgene and human FVIII antigen also were detected in the adipose tissue by RT-PCR and immunofluorescence, respectively, on day 14. Emergence of anti-human FVIII antibodies 14 days after the injection of SIVhFVIII may explain the disappearance of human FVIII from the circulation. These results suggest that transduction of the adipocytes with vectors carrying the human FVIII gene may be potentially applicable for gene therapy of hemophilia A.*
Gene Therapy (2004) **11,** 253–259. doi:10.1038/sj.gt.3302174

Keywords: *adipocyte; simian immunodeficiency virus vector; hemophilia*

Introduction

Hemophilia A is an inherited X-linked lifelong bleeding disorder caused by abnormality in the coagulation factor VIII (FVIII) gene.[1,2] The genetic abnormalities result in deficiency of FVIII, which in turn creates a bleeding diathesis, such as life-threatening intracranial bleeding and bleeding in joints and muscles. Hemophilias occur as mild, moderate, or severe, depending on the blood FVIII level of 6% or more, 2–5%, or 1% or less. The current standard therapy is intravenous (i.v.) injection of human plasma-derived FVIII or recombinant FVIII. Aside from certain specific situations, such as preoperative factor coverage, i.v. infusion of FVIII is usually used to treat acute bleeding episodes and prophylactic FVIII i.v. infusion is not recommended. However, maintaining of blood FVIII levels to more than 5% of the normal FVIII concentration may result in significant clinical improvement. Furthermore, if one can increase FVIII levels to more than 1% in severe hemophilia patients, they may have significantly fewer bleeding episodes and improved quality of life. Recombinant FVIII products are now commercially available, but may not be completely free from pathological substances such as prions or as yet unknown viruses. In this regard, gene therapy is being explored as the next generation therapy for hemophilia patients.[1,2]

Adipocytes are terminally differentiated and nondividing cells. They not only store excess energy in the form of fat but also synthesize and secrete a variety of biologically active molecules such as leptin, adiponectin, cytokines, and plasminogen activator inhibitor-1 to the circulation.[3] Subcutaneous adipose tissues are readily accessed for vector administration. In addition, the adipose tissue can be removed surgically if necessary. These characteristics present attractive features of adipocytes for therapeutic gene therapy. In the present study, we use SIVagmTYO1-based vectors to show that SIVagmTYO1vectors can transduce adipocytes *in vitro* and *in vivo*, resulting in therapeutic gene expression, an expression mode that may be applicable to hemophilia gene therapy.

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Received 17 March 2003; accepted 3 September 2003

Results

Transduction of adipocytes with SIVeGFP and SIVhFVIII in vitro

To assess the *in vitro* transduction efficiency of adipocytes with the SIV vector, human adipocytes were cultured in the presence of increasing concentrations of SIVeGFP for 48 h. After transduction, cells were washed and incubated in medium for 72 h. As shown in Figure 1a (phase-contrast view), cells containing intracellular lipid droplets represent the typical adipocyte morphology. The fluorescent microscopy image showed that eGFP was expressed in lipid droplet-containing adipocytes (Figure 1b). Flow cytometry analysis (Figure 1c) of these cells showed that eGFP expression in human adipocytes increased in a dose-dependent manner. Approximately 62% of adipocytes were efficiently transduced with SIVeGFP at MOI 5×10^3 vg/cell (100 transduction units/cell). We also assessed human FVIII production in the transduced human adipocytes. Cells were incubated in the presence of increasing concentrations of SIVhFVIII. The supernatants were harvested after various incubation times and human FVIII antigen levels were quantified by ELISA. FVIII production from the human adopocytes started on day 5 and increased in a dose- and time-dependent manner (Figure 2). After

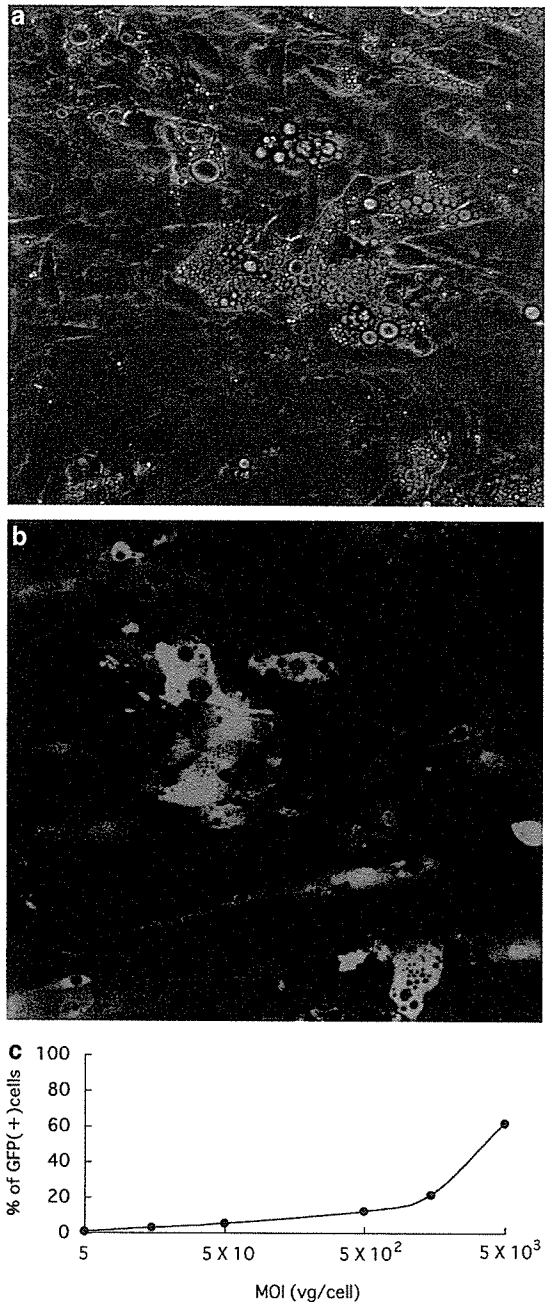

Figure 1 *Transduction of human white adipocytes with SIVeGFP. Human white adipocytes (6.7×10^4cells/well) were incubated with increasing concentrations of SIVeGFP for 48 h. After incubation, cells were washed with PBS and incubated in media for 72 h (a, phase contrast). Expression of eGFP was visualized by fluorescence microscopy (b) and flow cytometry (c). The percentages of transduced cells expressing eGFP are shown (c) (mean, n = 2).*

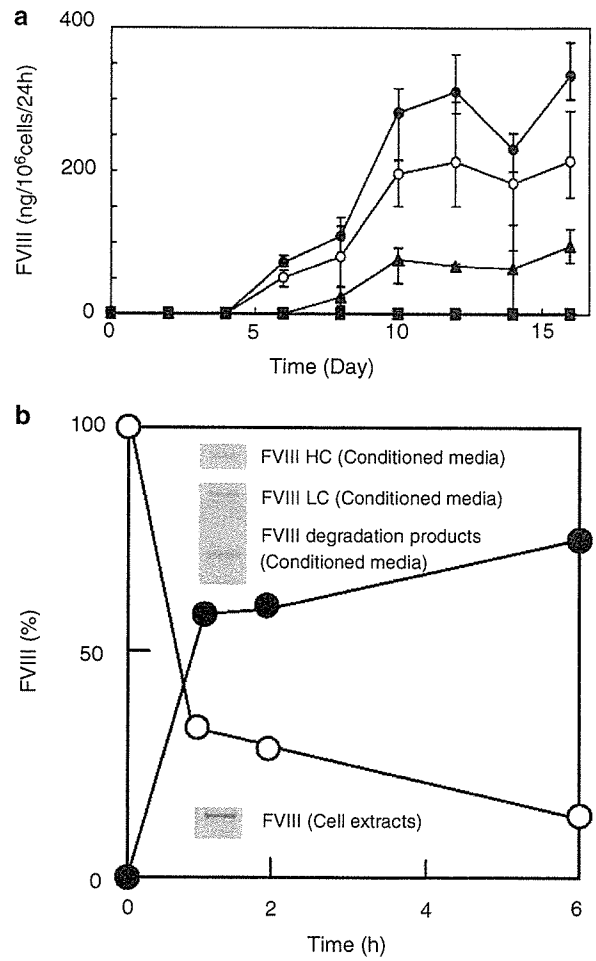

Figure 2 *Transduction of human white adipocytes by SIVhFVIII. (a) Human white adipocytes (6.7×10^4 cells/well) were incubated with increasing concentrations of SIVhFVIII (■, 2×10^2 vg/cell; ▲, 6.5×10^2 vg/cell; ^, 2×10^3 vg/cell; ●, 6.5×10^3 vg/cell). After 48 h, cells were washed with PBS and cultured for 14 days. Supernatants were harvested and human FVIII antigen quantified by ELISA, as described in Materials and methods (mean ± s.d., n = 3). (b) Pulse-chase experiments for FVIII production in human adipocytes (5×10^5 cells) were carried out on day 7 after transduction with SIVhFVIII (6.5×10^3 vg/cell). Amounts of [^{35}S]-labeled FVIII in the conditioned media (●) and in the cell extracts () were quantified as described in Materials and methods. Insets show the pertinent portion of the autoradiography of [^{35}S]-labeled FVIII in the cell extracts after pulse labeling and [^{35}S]-labeled FVIII species (heavy chain (HC); light chain (LC); degradation products) in the conditioned media after 1 h chase incubation.*

transducing cultured human adipocytes with SIVhFVIII at MOI 6.5×10^3 vg/cell, 320 ± 39.8 ng of human factor VIII was produced from 10^6 adipocytes during a 24 h *in vitro* incubation. To study the secretion of FVIII from transduced adipocytes, pulse-chase experiments were performed. Approximately 53% of human FVIII, expressed in cultured adipocytes, was secreted from adipocytes during 1 h incubation periods, suggesting that adipocytes could secrete expressed FVIII efficiently. FVIII molecules identified in the conditioned media consisted of the heavy chain, the light chain, and degraded FVIII products, and the presence of these FVIII species in the conditioned media were consistent with the previous report.[4]

Transduction of the adipose tissue by SIVlacZ in vivo

To explore the possibility that the SIV vector can transduce adipocytes *in vivo*, SIVlacZ vectors were injected into the subcutaneous adipose tissue of 8-week-old *db/db* mice. Wild-type mice have adipose tissue in the mesenterium and peritesticular regions, but they are generally lean and do not have enough subcutaneous adipose tissues for vector injection. NOD/SCID mice are used frequently for gene therapy research because of their immunodeficiency, but they also have little subcutaneous adipose tissues. Among several types of obese mice used for metabolic disease research, *db/db* mice are well characterized, obese, and diabetic. They become obese by accumulating fat in the subcutaneous and visceral adipose tissues after 4 weeks of age. Thus, *db/db* mice are appropriate for studying *in vivo* transgene expression from subcutaneous adipose tissue. At 2 weeks after the injection, the adipose tissues were excised and processed for detection of β-galactosidase activity. As shown in Figure 3a, the adipose tissue was stained blue homogenously after the X-gal staining in the macroscopic view. In the histology sections of the adipose tissue, β-galactosidase activity was detected in the adipocytes of *db/db* mice (Figure 3b, c). These data suggest that SIV vectors are capable of transducing adipocytes *in vivo* in mice.

Plasma human FVIII levels in SIVhFVIII-injected db/db mice

To evaluate *in vivo* production of human VIII from adipocytes, SIVhFVIII was injected into subcutaneous adipose tissues of *db/db* mice. Mouse plasma was obtained on days 0, 4, 7, 11, 14, 18, 21, and 25 after vector injection and human FVIII levels in mouse plasma were quantified by an ELISA that recognizes only human FVIII. Plasma human FVIII levels (closed circle) increased to 1.8 ng/ml on day 11, but human FVIII antigen was not detectable in the circulation on day 14 after vector injection (Figure 4). Since *db/db* mice are immunocompetent, they may develop antibodies to human FVIII expressed *in vivo*. To explore the possibility that disappearance of human FVIII from the mouse circulation was caused by the presence of antibody against human FVIII in *db/db* mice, a solid-phase EIA for detection of *db/db* mouse antibody to human FVIII was carried out. As shown in Figure 4, anti-human FVIII antibodies (triangle) were detected in *db/db* mouse plasma obtained on day 14 and the levels increased gradually to 6.9 μg/ml by day 25.

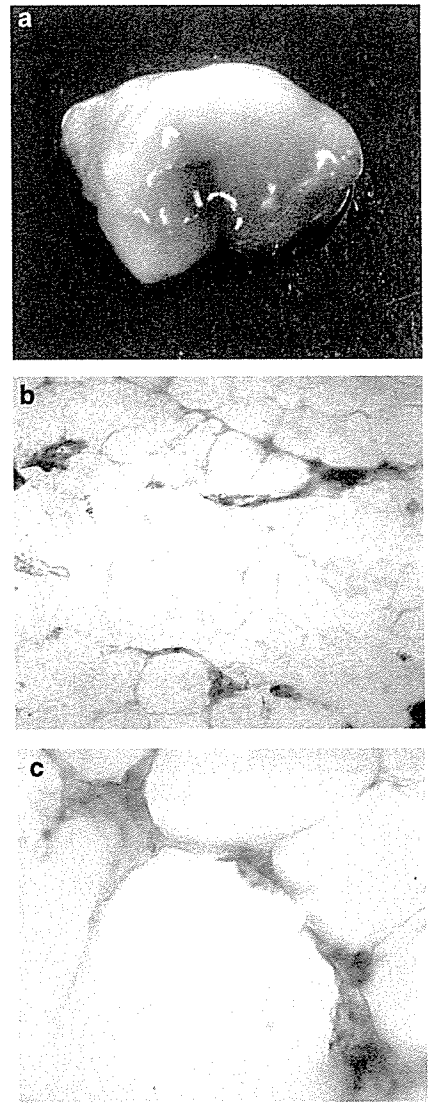

Figure 3 Transduction of the adipose tissues of db/db mouse by SIVlacZ. The SIVlacZ vector (5×10^7) was diluted in PBS and was injected into the subcutaneous adipose tissues of db/db mice. The subcutaneous adipose tissues were excised on day 14 after injection. Tissues were processed for detection of β-galactosidase activity, as described in Materials and methods. The adipose tissue (macroscopic view) was homogenously stained blue (a). β-galactosidase activity was observed in the adipocytes, as reflected by the blue staining in the histology sections (b, c).

Detection of FVIII transcripts in the adipose tissue of db/db mice

To assess the expression of transgenes in the adipose tissue of *db/db* mice, the adipose tissues were excised on day 14 after injection and subjected to reverse transcription-polymerase chain reaction (RT-PCR) analysis for detection of human BDD-FVIII transcripts using human FVIII or mouse GAPDH-specific primers. As shown in Figure 5, human FVIII transcripts were detected in the adipose tissue from the SIVhFVIII-injected mice, but not in the adipose tissue from SIVlacZ-injected mice. Mouse GAPDH transcripts were detected in both RNA preparations. These data suggest that the human FVIII transgene was expressed in the mouse adipose tissue.

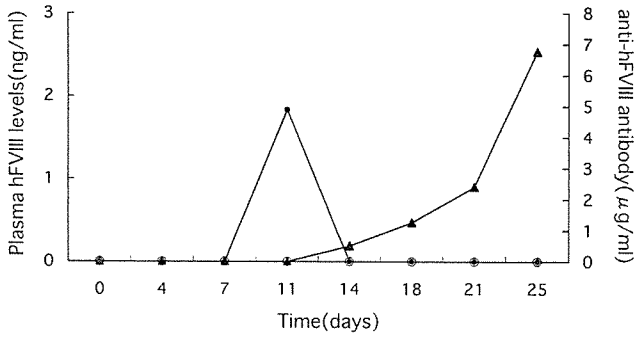

Figure 4 Plasma human FVIII levels in SIVhFVIII-injected db/db mice. Peripheral blood was obtained from SIVhFVIII-injected mice or the SIVlacZ-injected control mice on days 0, 4, 7, 11, 14, 18, 21, and 25 after injection. Human FVIII concentrations in plasma of db/db mice who received SIVhFVIII (closed circle) or SIVlacZ (open circle) injection were determined by ELISA (mean, n = 2). Anti-human FVIII antibodies present in mouse plasma (closed triangle) were quantified by the solid phase EIA as in described in METHODS.

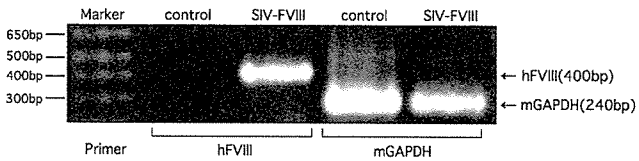

Figure 5 RT-PCR analysis of adipose tissue-derived RNA. RNA was isolated from the murine adipose tissue on day 14 after vector injection. A measure of 100 ng of RNA was subjected to RT-PCR with specific primer pairs for the human BDD-FVIII transcript (human FVIII) or for the mouse GAPDH transcript (mouse GAPDH). Amplified products were analyzed on 2% agarose gels followed by ethidium bromide staining (control, RNA isolated from the SIVlacZ injected adipose tissue; SIVhFVIII, RNA isolated from the SIVhFVIII-injected adipose tissue).

Detection of human FVIII expressed in the adipose tissue of db/db mouse

Mouse adipose tissues were processed for detection of human FVIII antigen, and imaged by immunofluorescence (Figure 6). Human FVIII was observed in adipocytes isolated from SIVhFVIII-injected mice (left), but not in cells from mice that received the SIVlacZ vector (right). These data confirm the notion that the human FVIII was produced from the SIVhFIII-transduced cells *in vivo*.

Discussion

The subcutaneous adipose tissue has attractive features for genetic therapy, such as easy accessibility, active biosynthesis and secretion behavior, and high vascularity.[3] However, adipocytes are terminally differentiated and nondividing cells with a characteristic phenotype expression. A variety of viral and nonviral vectors are used to transduce cells *in vitro* and *in vivo* for gene delivery, but it is still rather difficult to transduce nondividing cells efficiently. Adenoviral vectors are able to transduce nondividing cells including adipocytes,[5,6] but transgene expression by adenoviral vectors is thought to be transient and they are highly immunogenic. In contrast, pseudotyped lentiviral vectors are versatile enough to stably transduce various types of cells including nondividing cells, and were not as immunogenic as the adenoviral vectors.[7–10] But transduction of adipocytes with lentiviral vectors had not been studied yet. There are safety concerns in utilizing HIV-1-based lentivirus vectors for gene therapy clinical trials. In this regard, simian immunodeficiency virus agmTYO1 (SIVagmTYO1)-based vectors are of particular interest. SIVagmTYO1 is an HIV-related lentivirus isolated from the African green monkey and shown to be nonpathogenic to both their natural hosts and to experimentally inoculated Asian macaques.[11] Additionally, due to the use of contaminated blood products, some hemophilia patients are HIV-1 carriers. If an HIV-1-based vector is administered to such patients, the replication-competent lentivirus particles carrying the therapeutic gene may be generated by homologous recombination between the recombinant HIV vector and the wild-type HIV genome. The packaging signal in the HIV vector sequence may be another factor contributing to production of replication-competent lentivirus particles. From this perspective, then, a SIV vector based on the SIVagm TYO1 strain may be a better vehicle for hemophilia gene therapy because SIVagm TYO1 has less than 60% genomic sequence similarity to HIV-1.

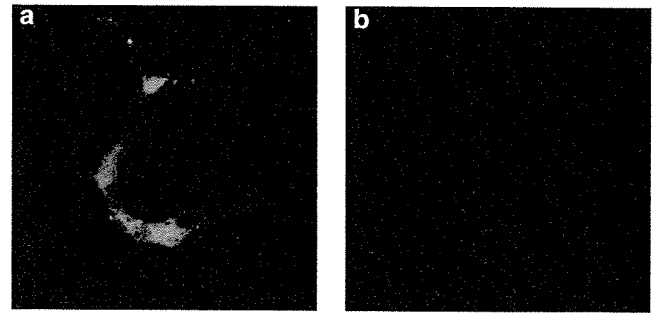

Figure 6 Detection of human FVIII antigen in mouse adipose tissue. The adipose tissue of SIVhFVIII injected db/db mice (a) or SIVlacZ-injected mice (b) were subjected to immunofluorescence staining for human FVIII antigen. Tissue sections were incubated with sheep anti-human FVIII polyclonal antibodies. The bound antibodies were detected by Alexa-Fluor488-conjugated secondary antibody and visualized using a fluorescence microscope (E800, Nikon Co Ltd, Tokyo, Japan).

We have shown that SIV vectors carrying the eGFP, the lac Z, or a therapeutic gene can transduce cultured human adipocytes *in vitro* and mouse adipose tissue *in vivo*. Transduction of the human adipocytes by the SIVeGFP vector *in vitro* was dose-dependent and appeared to be efficient. Production of human FVIII (320 ± 39.8 ng/10^6cells/24 h) from transduced adipocytes at MOI 6.5×10^3 vg/cell *in vitro* was considerable and efficient, raising the possibility of achieving therapeutic levels of plasma FVIII in mice if 10^6 adipocytes were transduced by SIVhFVIII efficiently *in vivo*. Thus, SIVhFVIII 4×10^9 vg was injected to the mouse subcutaneous adipose tissue. Human FVIII was detected in the mouse plasma and this human FVIII level was approximately 1–2% of the normal FVIII level of normal human subjects. The FVIII levels achieved in mice were relatively low, but such increase of the FVIII level would develop clinical effects in hemophilias such as decrease

of bleeding episodes and of the use of FVIII concentrates. Data on clinical trials of hemophilia A gene therapy support this notion.[12] However, this human FVIII level in mouse plasma was lower than that expected from the observed *in vitro* production rate. One contributing factor may be the shorter half-life of human FVIII in mice compared to humans. The half-life of injected human FVIII in mice is approximately 1 h,[13] whereas the half-life in hemophilia patients is closer to 8–12 h. Another possibility may be the inefficient transduction of adipocytes *in vivo* because the vector-containing solution might not diffuse throughout the adipose tissues, resulting in less adipocytes being exposed to the viral vectors. It is also possible that transduction of mouse adipocytes by the SIV vector is less efficient than that of human cells. Production of human FVIII from differentiated 3T3-L1 cells, transduced with the SIVhFVIII vector, was significantly lower than that from human adipocytes (not shown). The observation that anti-human FVIII antibodies were present in the mouse plasma obtained on day 14 and subsequently increased to 6.9 μg/ml by day 25 suggested that human FVIII was rapidly cleared from the mouse circulation by the formation of immune complexes after day 14. Thus, human FVIII was secreted into the circulation and was recognized by the *db/db* mouse immune system. The notion that human FVIII was synthesized in the adipocyts *in vivo* is also supported by detection of human FVIII gene transcripts and products in the adipocytes. In conclusion, adipose tissue is easily accessible and is an appropriate target for gene delivery. Recombinant SIV vectors may be applicable for adipocyte-targeted gene therapy for hemophilia A.

Materials and methods

Cell culture

Human white adipocytes prepared in 24-well culture plates were purchased from Zen-Bio Inc. (Research Triangle Park, NC, USA). These cells were differentiated from preadipocytes isolated from the adipose tissue of 49-year-old healthy subjects undergoing liposuction surgery with informed consent. The cells were shown to express leptin, CCAAT/enhancer-binding protein α, peroxisome proliferator-activated receptor γ, and STATs.[14,15] Human adipocytes were cultured in DMEM/HAM F10 medium supplemented with 3% fetal bovine serum, 15 mM HEPES, biotin (33 μM), pantothenate (17 μM), insulin (100 nM), dexamethazone (1 μM), penicillin 100 U/ml, streptomycin (100 μg/ml), and amphotericin B (0.25 μg/ml) (Zen-Bio Inc.). 3T3-L1 cells (ATCC) were cultivated in DMEM containing 10% FBS. Differentiation of 3T3-L1 cells to adipocytes was carried out in the medium containing dexamethasone, insulin, and 1-methyl-3 isobutylxantine, as described previously.[16]

Production of SIVagm vectors

Human FVIII cDNA spanning the entire coding region was a generous gift from Dr JA van Mourik (VU University Medical Centre).[17] As the B domain is excised from other FVIII domains upon activation by thrombin and is not essential for coagulation activity of FVIIIa, coding for the B domain was deleted from the human FVIII cDNA by PCR-based mutagenesis (BDD FVIII cDNA), as described previously.[18] The deletion was confirmed by sequencing. The characteristics and production of SIVagm vector used in this study were described previously.[19] Self-inactivating SIVagm vectors are pseudotyped with vesicular stomatitis virus glycoprotein G (VSVG). We constructed a series of gene transfer vectors to express the eGFP gene, the lacZ gene, and the hBDDFVIII gene driven by the cytomegalovirus (CMV) promoter. To produce SIV vectors, 293T cells were transfected with the packaging vector, the gene transfer vector, and pVSVG (Clontech), as described previously.[19] Transduction units of SIVeGFP were determined by infection of SIVeGFP to 293T cells, followed by determination of eGFP expression by FACS analysis. RNA dot blot analysis was performed to quantify the amount of SIVagmTYO1vector genome of vector preparations. When SIVhFVIII was produced in 293T cells, SIVeGFP also was prepared simultaneously and the transduction unit of SIVeGFP and the amount of vector genome were determined. The SIVeGFP preparation was used as the standard to estimate the transduction units of SIVhFVIII.

In vitro culture and transduction of human adipocytes

For transduction, increasing concentrations of the SIV vector were added to human adipocyte cell monolayers in 24-well culture plates, and the cells were incubated at 37°C for 48 h in the presence of 5% CO_2. After incubation, the medium was harvested and changed, according to the manufacturer's instruction. SIVeGFP-transduced adipocytes were analyzed for eGFP expression by fluorescence microscopy and flow cytometry, and the conditioned medium of SIVhFVIII-transduced adipocytes was harvested and subjected to FVIII ELISA. To study the secretion of FVIII from adipocytes transduced with SIVhFVIII, pulse-chase experiments were performed. SIVhFVIII-transduced adipocytes were cultured for 30 min in methionine-deficient DMEM (GIBCO-Invitrogen Japan, Tokyo, Japan) containing 3700 kBq/ml [^{35}S]-methionine (NEN Life Science Products, Inc., Boston, MA, USA), and then cultured in the complete medium (DMEM). After various incubation time periods, conditioned medium and cell extracts were prepared. To isolate [^{35}S]-labeled FVIII molecules, the conditioned media and cell lysates were subjected to immunoprecipitation using sheep polyclonal antibodies against human FVIII and protein A-coupled Sepharose CL-4B (Amersham Pharmacia Biotech, UK). [^{35}S]-labeled FVIII was analyzed by SDS-PAGE followed by autoradiography, and quantified using an image analyzer BAS 2000 (Fujifilm, Tokyo, Japan), as described.[20]

Mice

Experimental *db/db* mice (C57BL/KsJ-db/db) are well characterized obese and diabetic mice caused by the genetic abnormality of the leptin receptor gene,[21] and were purchased from Japan SLC Inc. (Hamamatsu, Japan). The *db/db* mice were kept in a clean P3-level experimental room, and were maintained on a sterile diet and given autoclaved water.

Transduction of mouse adipose tissues by SIV vectors

SIV vectors carrying either the lacZ gene or the hFVIII gene were diluted in PBS and injected into the subcutaneous adipose tissue of the mice. Peripheral

blood (100 μl) was collected from mouse tail veins into tubes containing heparin. Platelet poor plasma was prepared by centrifugation of the peripheral blood at 1000 g for 15 min, and subjected to FVIII ELISA. Some mice were killed on day 14 after the injection for detection of transcripts and products of the transgenes.

Enzyme immunosorbent assay (ELISA) for human FVIII antigen

Since human FVIII clotting activity could not be quantified directly in the *db/db* mice because of the presence of endogenous murine FVIII, human FVIII expressed in *db/db* mice was quantified by a human FVIII-specific ELISA, as described previously.[22] Briefly, 96-well microtiter plates (Costar, Cambridge, MA, USA) were coated with 1 μg/ml mouse monoclonal antibody to human FVIII (Chemo-Sero Institute, Kumamoto, Japan). After blocking with 5% casein in PBS, mouse plasma samples or pooled normal human plasma in Tris-buffered saline (TBS) containing 0.1% Tween 20, 1% casein were added. After 16 h incubation at 4°C, human FVIII bound to the plates was detected with sheep anti-human FVIII polyclonal antibodies (Cedarlane Laboratories Ltd, Homby, Ontario, Canada) and horseradish peroxidase-conjugated rabbit anti-sheep IgG.

Detection of anti-human FVIII mouse antibody

Microtiter plates were coated with purified human FVIII in PBS for 16 h. After blocking with 5% casein, FVIII-coated microtiter plates were incubated with mouse plasma at 10–1000-fold dilutions or monoclonal antibodies raised against human FVIII as the standard. Mouse IgG bound to human FVIII was detected by HRP-conjugated anti-mouse IgG, followed by incubation with HRP substrate ABTS (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD, USA).

Detection of β-galactosidase and human FVIII in the mouse adipose tissue

Mouse adipose tissues were obtained from *db/db* mice. The adipose tissues were fixed with 2% paraformaldehyde in PBS for 5 min, washed with PBS, and then incubated in PBS containing 1 mg/ml X-gal, 2 mM $MgCl_2$, 5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$, 0.01% Na deoxycholate, 0.1% Triton X-100 for 1 h. The tissues were again washed, incubated with PBS containing sucrose (10–30%), and frozen with OTC compound (Tissue-Tek, Miles Inc., Elkhart, IN, USA) in dry ice/ethanol. Tissue sections were made at −35°C and attached to polylysine-coated glass slides. For the immunofluorescence study, the adipose tissues were fixed with 4% paraformaldehyde in PBS for 2 h at 4°C, incubated with PBS containing sucrose (10–30%), and then frozen in the presence of OCT compound in dry ice/ethanol. Sections were prepared from frozen tissues at −35°C and attached to polylysine-coated glass slides. For the detection of human FVIII, tissue sections were blocked with 1% bovine serum albumin in PBS. Samples were incubated with polyclonal anti-human FVIII antibody at 4°C for 16 h. After washing in PBS, cells were incubated with donkey anti-sheep IgG antibody conjugated with Alexa488 (Molecular Probes, Eugene, OR, USA) at 4°C for 16 h for visualization of human FVIII by fluorescence microscopy.

Detection of the BDD-FVIII transcript by RT-PCR

RNA was isolated from the adipose tissue by the acid-guanidine method and was reverse transcribed to cDNA using reverse transcriptase (Superscript, Invitrogen Japan, Tokyo, Japan) and oligo-(dT) primers in a 20 μl mixture (QIAGEN Japan, Tokyo, Japan) after DNase I (Amplification grade, Invitrogen) treatment. Subsequent PCR amplification was carried out with 1 μl of cDNA solution in a 50 μl reaction mixture containing 5 U of Taq polymerase, 10 mmol/l Tris-HCl (pH 8.5), 50 mM KCl, 1.5 mM $MgCl_2$, and 100 μM dNTPs in the presence of specific primer pairs (200 nM) designed to amplify the DNA fragments derived from the transcript of the BDD-FVIII transgene. Each PCR cycle consisted of denaturation at 94°C for 15 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s. The PCR products were analyzed by agarose gel electrophoresis. Authenticity of PCR products was confirmed by their molecular sizes after agarose gel electrophopresis, and by sequencing. The primer sequences for human FVIII are ATTGGAG-CACAGACTGACTT and ATATGGTATCATCATAGTCA (400 bp). Primers for mouse GAPDH were purchased from R&D Systems, Inc. (Minneapolis, MN, USA).

Acknowledgements

We thank Dr JA van Mourik for the full-length FVIII cDNA and Dr Nakagaki (Chemo Sero Institute) for monoclonal antibodies to human FVIII. We also thank Dr DJ Stearns-Kurosawa (IdEst, Inc) for critical reading and editing of this manuscript, and Ms Fumino Muroi for technical assistance. This work is supported by Grants-in-aid for Scientific Research #12670687 to JM and #13671078 to SM from the Ministry of Education and Science, and by Health and Labour Sciences Research Grants for Research on HIV/AIDS to KO and to YS from the Ministry of Health, Labour and Welfare.

References

1 Hoyer LW. Hemophilia A. *N Engl J Med* 1994; **330**: 38–47.
2 Kay MA, High K. Gene therapy for the hemophilias. *Proc Natl Acad Sci USA* 1999; **96**: 9973–9975.
3 Mohamed-Ali V, Pinkney JH, Coppack SW. Adipose tissue as an endocrine and paracrine organ. *Int J Obes Relat Metab Disord* 1998; **22**: 1145–1158.
4 Kaufman RJ, Wasley LC, Dorner AJ. Synthesis, processing, and secretion of recombinant human factor VIII expressed in mammalian cells. *J Biol Chem* 1988: 6352–6362.
5 Levine JA, Eberhardt NL, Jensen MD, O'Brien T. Adenoviral-mediated gene transfer to human adipocytes *in vitro*, and human adipose tissue *ex vivo* and rabbit femoral adipose tissue *in vivo*. *J Nutr Sci Vitaminol* 1998; **44**: 569–572.
6 Nagamatsu S *et al*. Adnovirus-medicated proproinsulin gene transfer into adipose tissues ameliorates hyperglycemia in obese diabetic KKA^y mice. *FEBS Lett* 2001; **509**: 106–110.
7 Miyoshi H *et al*. Transduction of human CD34+ cells that mediate long-term engraftment of NOD/SCID mice by HIV vectors. *Science* 1999; **283**: 682–686.
8 Chen W *et al*. Lentiviral vector transduction of hematopoietic stem cells that mediate long-term reconstitution of lethally irradiated mice. *Stem Cells* 2000; **18**: 352–359.
9 Woods NB *et al*. Lentiviral gene transfer into primary and secondary NOD/SCID repopulating cells. *Blood* 2000; **96**: 3725–3733.